BIOTIN CARBOXYLASES

A thesis submitted

by

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CONTENTS

CHAPTER 3

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material that has been previously published or written by another person, except where due reference is made in the text.

GREGORY J. GOODALL.

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ABBREVIATIONS

In addition to those accepted for use in the *Biochemical Journal*, the following abbreviations are used in this thesis:

DTE	dithioerythritol
BCCP	biotin carboxyl carrier protein
oATP	periodate oxidation product of ATP
o-dephosphopropionyl-CoA	periodate oxidation product of dephospho-propionyl-CoA
SDS	sodium dodecyl sulphate

SUMMARY

1. A method has been developed for purifying propionyl-CoA carboxylase from sheep liver mitochondria. The method involves fractionation with ammonium sulphate and polyethylene glycol, followed by chromatography on DEAE-Sephacel and phenyl-Sepharose. Hexagonal bipyramidal enzyme crystals of 0.1 mm diameter have been obtained. The enzyme has been shown to contain two subunit types, with molecular weights of 58,000 and 72,000. The biotin prosthetic group resides in the larger subunit. The propensity of the enzyme for aggregating thwarted attempts to accurately measure its molecular weight, but "apparent" molecular weights in the range 670,000 to 840,000 were obtained using a variety of techniques.

2. Chemical modification experiments were undertaken in an attempt to determine to which subunit of propionyl-CoA carboxylase the substrates MgATP and propionyl-CoA bind. N-ethylmaleimide and the periodate oxidation products of ATP and dephosphopropionyl-CoA each bound to both subunits. In each case there was some protection against modification by both MgATP and propionyl-CoA, indicating that the two substrates bind at each other's binding sites. The results obtained were consistent with the hypothesis that the substrates bind in a crevice between two subunits of different type.

3. An assessment of the evolutionary relationships between 5 different biotin-dependent enzymes from various organisms

was made by comparing amino acid compositions. The β-methylcrotonyl-CoA carboxylase of *Achromobacter* was found to be closely related to the vertebrate pyruvate carboxylases. Pyruvate carboxylase from *Pseudomonas citronellolis* was found to be more distantly related to the vertebrate pyruvate carboxylases and to β-methylcrotonyl-CoA carboxylase. There was no evidence of any relationships between acetyl-CoA carboxylase, propionyl-CoA carboxylase, transcarboxylase or pyruvate carboxylase. However, the two subunits of propionyl-CoA carboxylase have very similar compositions. The same is true of the subunits of the pyruvate carboxylase from *Pseudomonas citronellolis* and the two larger subunits of transcarboxylase. There was no indication that subunits from different enzymes might be related.

4. Some of the factors that influence the location and rate of movement of the N-carboxybiotin moiety between the two sub-sites of pyruvate carboxylase were studied. The rate of carboxylation of the alternative substrate, 2-oxobutyrate, was measured at 0°C in an assay system where the isolated enzyme-[^{14}C]-carboxybiotin was the carboxyl group donor. The results are consistent with the hypothesis that the location of the carboxybiotin in the active site is determined by the presence of Mg^{2+}, acetyl-CoA and the oxo acid substrate. The presence of Mg^{2+} favours the holding of the complex at the first sub-site, whereas α-oxo acids induce the complex to move to the second sub-site. The allosteric activator acetyl-CoA exerts only a slight stimulation on the rate of translocation to the second

sub-site, and this stimulation arises from an increase in the dissociation constant for Mg^{2+}.

5. The mechanism of carboxyl group transfer, as catalysed by pyruvate carboxylase and propionyl-CoA carboxylase, was studied. It was found that oxo acids such as oxamate, glyoxylate and hydroxypyruvate induce the movement of carboxybiotin into the second subsite of pyruvate carboxylase, where hydrolysis of the carboxybiotin occurs. This was interpreted to be inconsistent with the mechanisms previously proposed for the carboxyl transfer reaction. It was shown that, unlike the pyruvate carboxylase reaction, there was no abortive hydrolytic pathway in the reaction catalysed by propionyl-CoA carboxylase, indicating that the two enzymes might use different catalytic mechanisms. However, a mechanism that is consistent with all the information available on biotin-dependent carboxyl group transfer reactions was proposed.

CHAPTER 1

INTRODUCTION

1.1 GENERAL PROPERTIES OF BIOTIN-DEPENDANT ENZYMES

1.1.1 The Different Enzymes and Their Reactions

The biotin-dependant enzymes carry out carboxylation or decarboxylation reactions. Each reaction proceeds through a carboxy-enzyme intermediate, with the carboxyl group attached to the enzyme prosthetic group, biotin. Of the nine known biotin-dependant enzymes, six catalyse an ATP-dependant carboxylation (of acetyl-CoA, propionyl-CoA, β-methylcrotonyl-CoA, geranyl-CoA, pyruvate and urea), two catalyze a decarboxylation reaction (of methylmalonyl-CoA and oxaloacetate) and one catalyses a transcarboxylation reaction. A full list of the enzymes and their reactions is shown in Table 1.1. Each of the reactions carried out by the biotin enzymes is the sum of two partial reactions, the first being the carboxylation of biotin and the second being the transfer of the carboxyl group to an acceptor substrate. These partial reactions are also shown in Table 1.1.

Various aspects of the biotin-dependant enzymes have been covered in reviews by Knappe (1970), Moss & Lane (1971), and Wood and Barden (1977), while other reviews deal with one or a few of the biotin enzymes. These include acetyl-CoA carboxylase (Lane *et al.*, 1974; Lane *et al.*, 1975), the acyl-CoA carboxylases (Alberts & Vagelos, 1972), pyruvate carboxylase (Scrutton & Young, 1972; Utter *et al.*, 1975) and transcarboxylase (Wood, 1976; Wood & Zwolinski, 1976).

1.1.2 The Role of Biotin

Biotin, which on occasions in the past has been referred to as "yeast growth factor", "protective factor X", "vitamin H", "coenzyme R", "protective factor against egg-white injury" and "biotin", consists of fused ureido and tetrahydrothiophene rings with a 5-carbon aliphatic side chain attached to the C-2 position of the tetrahydrothiophene ring (Fig. 1.1). The two rings are tilted with respect to each other into a boat-like configuration and the valeric acid side chain has a cis orientation with respect to the ureido ring, so that the distance between the side chain C-6 and the 3'-nitrogen of the ureido ring is 2.86 A° (De Titta *et al.*, 1976). This is sufficiently close to sterically hinder carboxylation at the 3'-nitrogen. It has been shown by Lynen and co-workers (Lynen *et al.*, 1959; Lane & Lynen, 1963; Wood *et al.*, 1963; Numa *et al.*, 1964) that both free and enzyme-bound biotin are carboxylated exclusively at the 1'-N. Guchhait *et al.* (1974b) have confirmed that 1'-N-carboxybiotin is the active intermediate by using the chemically synthesized compound as a substrate for both partial reactions of acetyl-CoA carboxylase.

Biotin is attached as a prosthetic group to each of the biotin enzymes by an amide linkage to the ε-amino group of a specific lysine residue. This reaction is catalysed by a single holoenzyme synthetase (McAllister & Coon, 1966) and requires ATP and Mg^{++} (Kosow *et al.*, 1962). The distance from the biotin C-2 to the α-carbon of the lysine is 14 A° (Gregolin *et al.*, 1968) so that the enzyme-bound biotin has the potential to translocate between separate

catalytic sites.

Visser and Kellogg (1978) have pointed out the similarities between biotin and lipoic acid, the prosthetic group which acts as an acetyl-group shuttle in the pyruvate dehydrogenase and oxoglutarate dehydrogenase complexes. These authors stress that biotin is a prosthetic substrate, with no catalytic role in the sense of stabilization of an intermediate, as other coenzymes have. It is the rest of the enzyme that provides catalysis. This explains the lack of success of the many model studies (Caplow, 1965; Caplow & Yager, 1967; Caplow, 1968; Bruice & Hegarty, 1970; Kluger & Adawadkar, 1976; Kohn, 1976; Visser & Kellogg, 1977; Tsuda *et al.*, 1979) designed to elucidate the chemistry of biotin carboxylation and decarboxylation.

1.1.3 Structural Features

Most of the biotin enzymes are large multi-subunit proteins with molecular weights greater than 400,000. A possible exception is methylmalonyl-CoA carboxylase, which has been partially purified from *Micrococcus lactilyticus* and is reported to have a molecular weight of 275,000 - 300,000 (Galivan & Allen, 1968). However, this is not a very reliable estimate as it was obtained from a comparison of the sedimentation in sucrose density gradients with two smaller marker proteins.

Transcarboxylase (E.C.2.1.3.1), which has been isolated from *Propionibacterium shermanii*, is the largest biotin-containing enzyme, with a molecular weight of 1.2×10^6 (Wood & Zwolinski 1976). It is composed of 30 subunits in all; 12 biotin-containing subunits with a molecular

weight of 12,000, 12 pyruvate-binding subunits with a molecular weight of 60,000, and 6 methylmalonyl-CoA-binding subunits (each with two active sites) with a molecular weight of 60,000. The methylmalonyl-CoA binding subunits form a hexameric core with three dimer-pairs of pyruvate-binding subunits at each end. One biotin subunit binds at each interface between a pyruvate-binding subunit and a methylmalonyl-CoA-binding subunit. The molecule also contains one metal ion (Co^{2+} or Zn^{2+}) per pyruvate-binding subunit. This subunit, in conjunction with the biotin-containing subunit, can catalyse a pyruvate-oxaloacetate exchange, while the core subunits plus the biotin-containing subunit catalyse a propionyl-CoA-methylmalonyl-CoA exchange.

Acetyl-CoA carboxylase (E.C.6.4.1.2) from *E. coli* is similar to transcarboxylase in that different activities are located on different subunits. The enzyme dissociates on purification into three components (Guchhait *et al.*, 1974a). The biotin carboxyl carrier protein (BCCP) is a dimer of two biotin-containing subunits with a molecular weight of 22,500. Biotin carboxylase, which can carboxylate free biotin or the BCCP from HCO_3^- and MgATP, is a dimer of two 50,000 dalton subunits. Carboxyltransferase catalyses the transfer of a carboxyl group from carboxyl-BCCP to acetyl-CoA and contains polypeptides of 30,000 and 35,000 daltons in an $\alpha_2\beta_2$ arrangement.

In the acetyl-CoA carboxylases of various animals the activities all appear to reside in a single large polypeptide of 230,000 - 250,000 daltons (Mackall & Lane, 1977; Hardie & Cohen, 1978; Tanabe *et al.*, 1975). Previous

observations of multiple smaller subunits were apparently due to proteolytic cleavage during purification. The enzyme is composed of two subunits, so that its molecular weight is about 500,000. The purified enzyme aggregates into polymeric filaments in the presence of tricarboxylic acids such as citrate and iso-citrate, which are allosteric activators of the enzyme.

Pyruvate carboxylase (E.C.6.4.1.1) from most sources (including bacterial, avian and mammalian) is a tetramer of apparently identical subunits each with a molecular weight of 110,000 - 130,000. In eukaryotes each subunit of the enzyme contains a tightly bound divalent metal ion. The enzymes with this type of structure are activated, to various extents, by acetyl-CoA. On the other hand, pyruvate carboxylase from *Pseudomonas citronellolis* has a different subunit composition (Barden *et al.*, 1975) and is not activated by acetyl-CoA (Seubert & Remberger, 1961). This enzyme has two types of subunit, of 54,000 and 65,000 daltons, in an $\alpha_4\beta_4$ arrangement (Cohen *et al.*, 1979).

The subunit size and arrangement of pyruvate carboxylase from *P. citronellolis* is similar to that of several other biotin enzymes; - propionyl-CoA carboxylase, β-methylcrotonyl-CoA carboxylase and geranyl-CoA carboxylase. In each case only the larger subunit contains biotin. The subunit molecular weights of these enzymes from several sources are listed in Table 1.2. It is unlikely that the observed polypeptides result from the proteolysis of a larger subunit as the two types of subunit are found when β-methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase are purified in the

presence of protease inhibitors (Lau *et al.*, 1979; this thesis Chapter 3).

1.1.4 Evolution of the Biotin Enzymes

There are several well-established protein "families", consisting of two or more proteins that have arisen from the divergent evolution of genes that originally came from the duplication of an ancestral gene. Some examples are myoglobin and hemoglobin, the pancreatic serine proteases, and, rather surprisingly, the milk protein α-lactalbumin and egg-white lysozyme. It has been suggested by Lynen (1974) and Wood & Barden (1977) that the biotin enzymes might also constitute such a family, in view of the similarities, of reaction and structure, that these enzymes share. There has also been the proposal that different biotin enzymes from the same organism may share common subunits, but this is not the case in those species which have been investigated (Fall *et al.*, 1975; Obermayer & Lynen, 1977).

The reactions catalysed by the nine biotin enzymes are shown in Table 1.1. The first six listed have the same first partial reaction involving the carboxylation of biotin from ATP and HCO_3^-. In the second partial reaction, four of these enzymes transfer the carboxyl group to an acyl-CoA substrate. In addition, transcarboxylase shares partial reactions with pyruvate carboxylase and propionyl-CoA carboxylase. As the partial reactions of transcarboxylase are catalysed by different subunits (Chuang *et al.*, 1975), it is conceivable that one subunit is related to pyruvate carboxylase and another subunit to propionyl-CoA carboxylase.

Transcarboxylase and *E. coli* acetyl-CoA carboxylase have three unifunctional subunit types, one to carry the biotin prosthetic group and one to catalyse each partial reaction (Chuang *et al.*, 1975; Guchhait *et al.*, 1974a). Several biotin enzymes have two types of subunit (see Table 1.2), while eukaryote pyruvate carboxylase and acetyl-CoA carboxylase have a single polyfunctional subunit type. Accordingly, Lynen (1975) has suggested that the various biotin enzymes might represent stages in the evolution of an enzyme system which originally carried out carboxylations with a single carboxyl-carrier protein interacting with several different enzymes. In the course of evolution an increased functional structuring of the cellular interior promoted the formation of multifunctional polypeptides, presumably by gene fusion.

There is certainly strong similarity in the region of the biotin prosthetic group of several different biotin enzymes. McAllister & Coon (1966) have shown that several holoenzyme synthetases, (the enzyme which attaches free biotin to newly-synthesized apo-carboxylases), do not discriminate between apo-propionyl-CoA carboxylase from rat liver, apo-β-methylcrotonyl-CoA carboxylase from *Achromobacter* and apo-transcarboxylase from *Propionibacterium shermanii*. Furthermore, biotin containing peptides from sheep, chicken and turkey pyruvate carboxylases, *P. shermanii* transcarboxylase and *E. coli* acetyl-CoA carboxylase have homologous amino acid sequences (Rylatt *et al.*, 1977; Maloy *et al.*, 1979; Sutton *et al.*, 1977). However, as Rylatt *et al.* point out, the similarity of the primary structure in a small region about

the biotin could have arisen by convergent evolution and the requirement for a recognition site by a single holoenzyme synthetase.

On the other hand, immunological studies designed to detect similar surface features among biotin enzymes have given negative results. Antibodies against rat liver pyruvate carboxylase do not inhibit propionyl-CoA carboxylase or acetyl-CoA carboxylase from the same source (Ballard *et al.*, 1970). Similarly, antibodies against yeast acetyl-CoA carboxylase do not cross react with yeast pyruvate carboxylase and vice versa (Sumper & Riepertinger, 1972); nor do antibodies against bovine kidney β-methylcrotonyl-CoA carboxylase cross react with bovine kidney propionyl-CoA carboxylase and vice versa (Lau *et al.*, 1979). Thus the relationships between the various biotin enzymes remain undetermined. These relationships are discussed further in Chapter 4.

1.2 PROPIONYL-CoA CARBOXYLASE

1.2.1 Distribution and Metabolic Significance

Propionyl-CoA carboxylase (E.C.6.4.1.3) has been reported to be present in a wide range of cell types, including bacterial (Olsen & Merrick, 1968), nematode (Meyer *et al.*, 1978), heart (Kaziro *et al.*, 1960), skeletal muscle (Davis *et al.*, 1980), liver (Halenz & Lane, 1960), leukocytes (Hsia & Scully, 1973), cultured amniocytes (Gompertz *et al.*, 1975), and cultured fibroblasts (Gravel *et al.*, 1977). Scholte (1969) has shown the enzymes from rat liver to be

located in the inner mitochondrial matrix. In contrast, the enzyme in the free-living nematode *Turbatrix aceti*, is cytoplasmic. This organism has no acetyl-CoA carboxylase, but instead uses propionyl-CoA carboxylase as a dual-purpose enzyme.

Propionyl-CoA arises from the oxidation of odd-chain fatty acids, the degradation of valine and isoleucine, and from propionate formed by fermentation by the gastrointestinal flora of ruminants and other animals. The product of the propionyl-CoA carboxylase reaction, S-methylmalonyl-CoA, is converted to its R-optical isomer by methylmalonyl-CoA racemase, and subsequently to succinyl-CoA by the vitamin B_{12} coenzyme dependant methylmalonyl-CoA mutase. Thus propionyl-CoA carboxylase is an intermediary in gluconeogenesis from propionate.

A defective or lowered level of propionyl-CoA carboxylase results in propionic acidaemia, a recessively inherited metabolic disorder. This disease is characterized by an intolerance to protein manifested by hyperammonaemia, ketoacidosis, suppression of maturation and release of cells from the bone marrow, and overwhelming, often lethal illness in infancy. Genetic complementation tests on fibroblast cell lines obtained from children with propionic acidaemia reveal two complementation groups (Gravel *et al.*, 1977) from which Wolf *et al.* (1978) predicted the multi-subunit nature of the human enzyme.

1.2.2 The Reaction

On the basis of isotope exchange studies (see below) the overall reaction catalysed by propionyl-CoA carboxylase (eqn. 3) can be partitioned into two partial reactions (eqns 1 and 2).

$$ATP + HCO_3^- + \text{ENZ-biotin} \overset{Mg^{2+}}{\rightleftharpoons} \text{ENZ-biotin-}CO_2 + ADP + Pi \quad (1)$$

$$\text{ENZ-biotin-}CO_2 + \text{propionyl-CoA} \rightleftharpoons \text{ENZ-biotin} + \text{methylmalonyl-CoA} \quad (2)$$

$$ATP + HCO_3^- + \text{propionyl-CoA} \rightleftharpoons ADP + Pi + \text{methylmalonyl-CoA} \quad (3)$$

The enzyme catalyses an isotopic exchange reaction between ATP and ^{32}Pi which requires ADP, HCO_3^- and Mg^{2+}; and an exchange reaction between [^{14}C]ADP and ATP, which requires Pi, HCO_3^- and Mg^{2+} (Kaziro *et al.*, 1960). In addition, the enzyme catalyses an exchange reaction between [^{14}C]propionyl-CoA and methylmalonyl-CoA in the absence of any other reaction components (Kaziro *et al.*, 1962). The ENZ-carboxybiotin intermediate prepared by incubating the enzyme with Mg^{2+}, ATP and HCO_3^- can be isolated at low temperatures (Kaziro & Ochoa, 1961) and the carboxyl group has been shown to be attached to 1'-N of the biotin prosthetic group (Lane & Lynen, 1963).

The binding of the acyl-CoA substrate to propionyl-CoA carboxylase involves the 3'-phosphate, adenine, and pantoyl moieties of the substrate (Hegre & Lane, 1966). Butyryl-CoA, acetyl-CoA, valeryl-CoA, crotonyl-CoA and isobutyryl-CoA are carboxylated at reduced rates by the enzyme from bovine liver (Lane *et al.*, 1960). In contrast, only ATP can serve as the nucleoside triphosphate substrate for the pig heart

enzyme (Tietz & Ochoa, 1959).

The overall reaction is stimulated by Cs^+, Rb^+, NH_4^+, and K^+ (Neujahr & Mistry, 1963). Giorgio & Plaut (1967) found a 7- to 9-fold stimulation of Vmax by K^+, using enzyme from bovine liver. The ATP-Pi, ATP-ADP, and propionyl-CoA-methylmalonyl-CoA isotopic exchange reactions were all stimulated by K^+. Edwards & Keech (1967) found the principal effect of K^+ on the pig heart enzyme to be a reduction of the apparent Km for HCO_3^- from 8 mM to 3 mM. Unlike the requirement for Mg^{2+}, an absolute requirement for K^+ could not be demonstrated.

So far there have been no reports of steady state studies on the kinetic mechanism of propionyl-CoA carboxylase, although some studies relating to the chemical mechanism have been reported. In experiments with $H_2^{18}O$ and $HC^{18}O_3^-$, Kaziro *et al.* (1962) showed that the oxygen for ATP cleavage is derived from bicarbonate, one bicarbonate oxygen appearing in the released orthophosphate and two in the free carboxyl group of methylmalonyl-CoA. These findings proved that HCO_3^-, and not CO_2, is the reactive species in the propionyl-CoA carboxylase reaction. In addition, when considered along with the requirement of Pi for the ADP-ATP isotopic exchange reaction, the ^{18}O results suggest that the formation of ENZ-carboxybiotin from ATP and HCO_3^- may proceed through a concerted reaction mechanism.

Using enzymically prepared [2-^{3}H]propionyl-CoA, Prescott & Rabinowitz (1968) could observe no tritium isotope effect on the reaction catalysed by propionyl-CoA carboxylase, indicating that C-H bond breaking is not involved in the

rate-limiting step. As tritium from ^3H_2O was not incorporated into propionyl-CoA in the absence of the reverse reaction, Prescott & Rabinowitz suggest a concerted mechanism for the transcarboxylation step. They also showed that carboxylation of propionyl-CoA occurred with retention of configuration about the α-carbon.

The concerted mechanism for the transcarboxylation step has been challenged recently by Stubbe & Abeles, who used β-fluoropropionyl-CoA as a substrate. Their initial report (Stubbe & Abeles, 1977), that the enzyme catalysed F^- elimination faster than ATP hydrolysis, was subsequently found to be in error (Stubbe *et al.*, 1980). Even so, a careful investigation failed to detect any carboxylation products of fluoropropionyl-CoA, which led Stubbe *et al.* to conclude that the enzyme can catalyse the abstraction of the α-proton without concomitant carboxylation of the substrate. Hence they proposed that the normal catalytic reaction proceeds via a carbanion intermediate.

1.2.3 Structure

Except for the early sedimentation studies of Kaziro *et al.* (1961), there has been little interest in the structure of propionyl-CoA carboxylase until recently. Kaziro *et al.* found a molecular weight of 700,000 for the pig heart enzyme and concluded that it was a tetramer on the basis of the biotin content. In 1978 Meyer *et al.* reported on an acyl-CoA carboxylase from the nematode *Turbatrix aceti*, which, though cytoplasmic, carboxylated propionyl-CoA faster than acetyl-CoA or butyryl-CoA (Meyer & Meyer, 1978; Meyer *et al.*,

1978). This enzyme was found to be a tetramer of 667,000 daltons, each protomer containing two distinct polypeptide chains with molecular weights 58,000 and 82,000. Subsequently the enzymes from *Mycobacterium smegmatis* (Henrikson & Allen, 1979) bovine kidney (Lau *et al.*, 1979) and human liver (Kalousek *et al.*, 1980) have been shown to have similar subunit compositions. Details can be found in Table 1.2.

1.3 PYRUVATE CARBOXYLASE

1.3.1 Distribution and Metabolic Significance

Pyruvate carboxylase (E.C.6.4.1.1) is found in most types of organism with the exception of higher plants and some bacteria, including *Escherichia, Aerobacter* and *Salmonella*. The most intensively studied forms of the enzyme have been isolated from sheep, chicken, rat and pig livers, yeast and *Pseudomonas citronellolis*. In vertebrates the enzyme is most abundant in liver and kidney, but also occurs in adipose tissue, brain, mammary tissue (Ballard *et al.*, 1970) skeletal muscle and heart (Davis *et al.*, 1980). In all species so far studied, pyruvate carboxylase is located mostly, if not wholly, in the mitochondrial matrix. This has been shown with human liver (Brech *et al.*, 1970), rat liver (Bottger *et al.*, 1969; Walter & Anabitarte, 1971), pigeon liver (Landriscinda *et al.*, 1970) and sheep liver (Taylor *et al.*, 1971).

Pyruvate carboxylase, in conjunction with PEP carboxykinase, circumvents the reverse reaction of pyruvate kinase, which under *in vivo* conditions is thermodynamically

unfavourable. Pyruvate and its precursors such as lactate, alanine, serine and cysteine thereby become gluconeogenic. Pyruvate carboxylase also plays an anaplerotic role, helping to regulate the level of citric acid cycle intermediates and it participates in lipogenesis. In vertebrates, the activity of the enzyme is regulated by acetyl-CoA, for which there is an almost complete dependence, while the yeast enzyme is stimulated four-fold by acetyl-CoA (Cooper & Benedict, 1968). In bacteria the affect of acetyl-CoA ranges from nil in *Pseudomonas* (Seubert & Remberger, 1961) to strong activation in *Bacillus* (Cazzulo *et al.*, 1969). The regulation of pyruvate carboxylase in gluconeogenic tissues has been reviewed by Barritt *et al.* (1976).

1.3.2 Reaction Mechanism

1.3.2.1 The overall reaction: The overall reaction catalysed by pyruvate carboxylase may be represented as:

$$MgATP^{2-} + HCO_3^- + \text{pyruvate} \xrightarrow[\text{acetyl-CoA}]{M^+,\ M^{2+}} MgADP^{-} + Pi + \text{oxaloacetate} \quad (1)$$

where M^+ and M^{2+} represent monovalent and divalent cations respectively. The free energy change of the reaction has been calculated by Wood *et al.* (1966) to be -0.5 ± 0.2 kcal at pH 7.0 and -1.8 ± 0.2 kcal at pH 8.0 (25°C, 5 mM Mg^{2+}).

The enzyme catalyses several isotopic exchange reactions, viz. ATP-^{32}Pi, ATP-[^{14}C]ADP, [^{14}C]pyruvate-oxaloacetate and $H^{14}CO_3^-$-oxaloacetate (Scrutton *et al.*, 1965; Scrutton & Utter, 1965). The isotopic exchange reaction between ATP and ^{32}Pi requires the presence of ADP, HCO_3^-, Mg^{2+} and acetyl-CoA, but not pyruvate or oxaloacetate. Conversely, the exchange

reaction between oxaloacetate and [^{14}C]pyruvate proceeds in the absence of any other components of the overall reaction. Hence the overall reaction can be regarded as the sum of two partial reactions;

$$MgATP^{2-} + HCO_3^- + \text{ENZ-biotin} \underset{\text{acetyl-CoA}}{\overset{M^+,\ M^{2+}}{\rightleftharpoons}} \text{ENZ-biotin-}CO_2^- + MgADP^- + Pi \qquad (2)$$

$$\text{ENZ-biotin-}CO_2^- + \text{pyruvate} \rightleftharpoons \text{oxaloacetate} + \text{ENZ-biotin} \qquad (3)$$

When the enzyme is incubated with ATP, Mg^{2+}, $H^{14}CO_3^-$ and acetyl-CoA an ENZ-[^{14}C]carboxybiotin intermediate can be isolated by gel filtration (Scrutton *et al.*, 1965; Rylatt, 1976), providing further support for the partitioning of the overall reaction into two discrete steps. The mechanism represented in equations (2) and (3) is referred to, in the terminology of Cleland (1970) as Ping-Pong Bi-Bi Uni-Uni. However, the steady state reaction does not follow a classical ping-pong pathway and has been a subject of some discussion for some time.

Kinetic studies of the pyruvate carboxylase mechanism have been undertaken using enzyme isolated from *Aspergillus niger* (Feir & Suzuki, 1969), rat liver (McClure *et al.*, 1971a, b, c), chicken liver (Barden *et al.*, 1972), pig liver (Warren & Tipton, 1974a, b, c, d), sheep kidney (Ashman & Keech, 1975) and sheep liver (Easterbrook-Smith *et al.*, 1976a, 1978). The sheep kidney, rat and chicken enzymes are reported to have a non-classical Bi-Bi Uni-Uni Ping-Pong reaction sequence, while the pig and sheep liver enzymes are found to have a sequential mechanism. This conflict may arise from the complicated nature of the kinetic properties

rather than real differences between the enzymes. I will discuss the two models and the evidence upon which each is based, and then describe how they might be reconciled.

1.3.2.2 The ping-pong model: Northrop, in 1969, found it necessary to propose a non-classical ping-pong mechanism in order to account for apparently conflicting results from his kinetic studies on the bacterial biotin enzyme, transcarboxylase. He found the kinetic patterns obtained from initial velocity studies at low substrate concentrations, to be typical of a ping-pong reaction sequence, with carboxybiotin-enzyme as an intermediate. However, kinetic patterns with inhibitors were inconsistent with such a mechanism. Northrop interpreted his results as indicating that the active centre of transcarboxylase has two separate and independant binding sites, or subsites, one for each of the partial reactions catalysed, and he constructed a mechanism which was a hybrid between a ping-pong mechanism and a random, ternary complex mechanism (Fig. 1.2). This led Northrop & Wood (1969) to propose that the two separate subsites were linked together by the covalently bound biotin acting as a mobile carboxyl-group carrier shifting the activated carboxyl group from one subsite to the other. The independence of the subsites was later confirmed by Chuang *et al.* (1975) who showed that the two partial reactions are carried out by different subunits. This has also been shown to be the case with *E. coli* acetyl-CoA carboxylase (Guchhait *et al.*, 1974a, b) and it may be that all biotin dependent enzymes have an active centre comprising two sub-sites.

Soon after Northrop reported the unusual mechanism of

transcarboxylase, McClure *et al.* (1971b) carried out a similar study on rat liver pyruvate carboxylase. They interpreted their results as indicating a "two-site ping-pong" mechanism analogous to the transcarboxylase mechanism, although there were some uncertainties. Double reciprocal initial velocity plots, with either HCO_3^- or MgATP as the variable substrate and pyruvate as the fixed variable substrate, should have been parallel, but were found to be intersecting. In both cases the replot of slope against $[pyruvate]^{-1}$ was concave downward, indicating some extra effect of pyruvate. Rather than reject the ping-pong mechanism, McClure *et al.* rationalized their results by assuming that pyruvate affects the affinity of the enzyme for bicarbonate. The effects of pyruvate are discussed further in Section 1.3.2.4.

In their study on the pyruvate carboxylase from chicken liver, Barden *et al.* (1972) avoided the complicating effects of pyruvate by only using low concentrations of that substrate. While this does simplify the interpretation, it can be misleading to ignore data that do not fit a model being tested. At best the model will be a simplified version of the true mechanism. (See Section 1.3.2.4). The otherwise comprehensive initial velocity and product inhibition studies of Barden *et al.* were reported to be consistent with a non-classical Ping-Pong Bi-Bi Uni-Uni kinetic mechanism.

Barden *et al.* have been criticized by Warren & Tipton (1974b) for assuming, on the basis of no published evidence, that HCO_3^- and MgATP randomly bind to the enzyme at equilibrium. Warren & Tipton using pig liver pyruvate carboxylase, found

that the steady-state binding of MgATP and HCO_3^- was an ordered process with the binding of MgATP preceding the binding of HCO_3^-. They point out that if this is the case for the chicken liver enzyme, it could not obey a non-classical ping-pong mechanism.

1.3.2.3 The sequential model: From initial velocity and product inhibition studies on pig liver pyruvate carboxylase, Warren & Tipton (1974b, c) have presented data which indicate a sequential mechanism. They found the order of substrate binding to be: Mg^{2+}, MgATP, HCO_3^- and finally pyruvate; while oxaloacetate was released before the random release of MgADP and Pi. Similar studies on the reverse reaction indicated a simple reversal of the order found in the forward reaction, although reciprocal plots involving oxaloacetate were not included in the study as they were downward concave. On the other hand double reciprocal plots involving pyruvate were linear up to at least 5 mM pyruvate, and when MgATP was varied at different fixed levels of pyruvate in the range 0.029 - 2.2 mM, the slope replot was linear. It would be interesting to see if this linearity is maintained over an even broader concentration range. As it stands there are no inconsistencies between the data and a sequential mechanism.

Warren & Tipton point out that the sequential mechanism provides an explanation for the low rate of the ATP-Pi isotopic exchange reaction (e.g., Scrutton *et al.*, 1965). The fact that the release of MgADP and Pi is only observed after the release of oxaloacetate suggests that enzyme-bound carboxybiotin (but not biotin) somehow "traps" the

MgADP and Pi at their respective binding sites. While this may well be the case, there are other possible limitations on the ATP-Pi isotopic exchange rate. Ashman & Keech (1975) have measured the rates of various reactions catalysed by pyruvate carboxylase. Some of the rates they measured, relative to the overall forward reaction, were: overall reverse reaction, 3.6%; pyruvate-oxaloacetate isotopic exchange, 47%; ATP-Pi isotopic exchange, 1.3%. As the overall reverse reaction is considerably slower than the pyruvate-oxaloacetate isotopic exchange reaction, the slow step in the reverse reaction must lie in the formation of ATP from carboxyenzyme, MgADP and Pi. This same slow step could limit the rate of the ATP-Pi isotopic exchange.

It is interesting to compare the initial velocity and product inhibition patterns obtained with pyruvate carboxylase from the various vertebrate sources studied so far. These are shown in Tables 1.3 and 1.4 respectively. Only intersecting initial velocity patterns were obtained by McClure *et al.* (1971b), Warren & Tipton (1974b) and Ashman & Keech (1975). On the other hand Barden *et al.* (1972) found four out of six plots to have parallel lines. Each of these four plots involves pyruvate as either the variable or the changing fixed substrate, and in each case only low concentrations of pyruvate were used in order to minimize the non-linear nature of the plots. At higher concentrations of pyruvate all of the plots would probably become intersecting, as was found by Ashman & Keech (1975).

When the product inhibition patterns are compared the only differences between species occur when pyruvate is the

varied substrate [Note that mixed inhibition is essentially the same as non-competitive inhibition (Cleland, 1970)]. Warren & Tipton found MgADP and Pi to be mixed competitive inhibitors with respect to pyruvate, which in a sequential mechanism suggests that ADP and Pi are the last to leave the enzyme. The other groups each found MgADP and Pi to be uncompetitive with respect to pyruvate. Although Ashman & Keech noted that in the case of Pi, the inhibition becomes non-competitive at high pyruvate concentrations. McClure *et al.* and Barden *et al.* only used low concentrations of pyruvate in their product inhibition studies, while Warren & Tipton do not indicate what range they used. It is possible that in each species the pyruvate concentration influences the product inhibition patterns as well as the initial velocity patterns. How this could occur is discussed in the next section.

1.3.2.4 Reconciling the models: One of the problems encountered by all who carried out initial velocity studies on pyruvate carboxylase was examined by Easterbrook-Smith *et al.* in 1976. The problem was the non-linear nature of primary or secondary plots where the pyruvate (or oxaloacetate) concentration was varied. The cause of this atypical kinetic behaviour was an abortive decarboxylation of ENZ-carboxybiotin which occurs at low pyruvate concentrations. Easterbrook-Smith *et al.* compared the rates of oxaloacetate and Pi production over a range of pyruvate concentrations and found that Pi release exceeded oxaloacetate production, especially at low levels of pyruvate. In the absence of pyruvate, negligible ATP hydrolysis occurred, indicating

that the binding of pyruvate was a prerequisite for the abortive hydrolysis. The important point here is that because reaction rates in the past had been measured in terms of oxaloacetate production, the alternative hydrolytic pathway had not been detected and included in model rate equations.

Easterbrook-Smith *et al.* (1978) subsequently measured initial rates in terms of Pi release and found intersecting lines in reciprocal plots where either MgATP or HCO_3^- was varied at several fixed concentrations of pyruvate. These results are incompatible with a mechanism in which Pi must be released before pyruvate can bind, as any irreversible step occurring before the binding of pyruvate would yield parallel lines in such reciprocal plots.

Easterbrook-Smith *et al.* also found intersecting reciprocal plots when MgATP was varied in the presence of alternative substrates (2-oxobutyrate, 3-fluoropyruvate and pyruvate). They claim that this refutes the ping-pong mechanism, but unfortunately they measured dicarboxylic acid formation without taking into account the alternative hydrolytic pathway. I have derived an initial velocity rate equation which describes the formation of oxaloacetate in a ping-pong mechanism with an alternative hydrolytic pathway included (See Fig. 1.3). The full derivation and equation are shown in an appendix (Section 1.3.7), but the important result is that the slope term now not only becomes dependent on the oxo-acid substrate concentration, but also contains rate constants which may be different for the various substrates (i.e., k_4, k_5, k_{-5}, and k_6). If the alternative hydrolytic pathway is significant under the conditions used by Easterbrook-Smith *et al.* (i.e., if k_4 is not >> k_5 or k_6), then their con-

clusion is not valid. This example demonstrates the need to reinterpret previous kinetic studies, taking into account the alternative pathways for decarboxylation of the carboxybiotin intermediate.

The fact that pyruvate carboxylase catalyses isotopic exchange reactions between MgATP and Pi and between MgATP and MgATP, both in the absence of pyruvate, proves that pyruvate binding is not essential for the release of MgADP or Pi. The sequential pathway described by Warren & Tipton (1974b) and Easterbrook-Smith *et al.* (1978) represents the preferred pathway under certain steady-state conditions, rather than a unique and obligatory pathway. Under different conditions (those of the various isotopic exchange reactions, for example) MgADP and Pi are released without the prior binding of pyruvate and conversely pyruvate can bind to carboxyenzyme in the absence of MgADP and Pi. The NMR and EPR studies of Mildvan *et al.* (1966) and of Reed & Scrutton (1974) show that pyruvate can also bind to the free form of the enzyme.

These different modes of substrate binding can be readily explained if pyruvate carboxylase, like transcarboxylase, has an active centre that comprises two functionally distinct subsites, the mobile biotin prosthetic group providing a link between the two (Scrutton *et al.*, 1973). Furthermore, when these different modes of substrate binding are incorporated into a single mechanism, the mechanism is analogous to the mechanism proposed for transcarboxylase by Northrop (1969) (Fig. 1.2) except for the additional pathway of abortive decarboxylation). Whether observed kinetic patterns appear to result from a ping-pong or a sequential mechanism will depend on the relative values of the rate constants in the alternative pathways, and on the experimental conditions.

High concentrations of pyruvate will favour a sequential pathway. Pyruvate carboxylases from different species may share the same overall mechanism but have different values for some rate constants, and hence produce different kinetic patterns.

1.3.3 Requirements for biotin carboxylation

The first partial reaction catalysed by pyruvate carboxylase is the carboxylation of enzyme-bound biotin from HCO_3^- and $MgATP^{2-}$;

$$HCO_3^- + MgATP^{2-} + \text{E-biotin} \underset{\text{acetyl-CoA}}{\overset{M^+,\ M^{2+}}{\rightleftharpoons}} \text{E-biotin}CO_2^- + MgADP + Pi$$

This reaction has a requirement for two divalent metal ions, which in the mitochondrion would probably be fulfilled by Mg^{2+}. One of these is complexed to ATP (Keech & Barritt, 1967). Free ATP^{4-} is unreactive and a competitive inhibitor. Some workers (e.g., Cazzulo & Stoppani, 1969) have suggested that the Mg^{2+} required in excess of ATP^{4-} does not activate pyruvate carboxylase, but merely ensures there is not an inhibitory concentration of ATP^{4-}. However, this interpretation is inconsistent with the initial velocity kinetics (Warren & Tipton, 1974b). Bais & Keech (1972) suggest that free Mg^{2+} induces a conformational change in the sheep kidney enzyme while Barden & Scrutton (1974) found that Mg^{2+} enhances the binding of MgATP and HCO_3^-, (and *vice versa*), to the chicken liver enzyme.

Monovalent cations are required for the first partial reaction, but only have a small effect on the pyruvate-oxaloacetate exchange reaction (McClure *et al.*, 1971c). The specificity for monovalent cations depends on the source of the enzyme (Scrutton & Young, 1972). When the overall reaction is monitored, the rat and chicken liver enzymes

are activated most by Rb^+, NH_4^+ and K^+ (McClure *et al.*, 1971b; Barden & Scrutton, 1974).

The species of CO_2 involved in the reaction is the bicarbonate anion. Initially this was shown for the reaction catalysed by propionyl-CoA carboxylase by Kaziro *et al.* (1962), who used $HC^{18}O_3^-$ and found that all three oxygen atoms are incorporated into products. Subsequently the bicarbonate anion was identified as the substrate of pyruvate carboxylase by Cooper *et al.* (1968). These investigators compared the incorporation of label from $^{14}CO_2$ and $H^{14}CO_3^-$, using low temperature (10°C) to slow down equilibration between the two.

Acetyl-CoA is required for carboxylation of chicken liver pyruvate carboxylase (Scrutton *et al.*, 1965). The rat liver enzyme, which catalyses an overall forward reaction in the absence of acetyl-CoA at 2% of Vmax, catalyses an acetyl-CoA-independent ATP-Pi exchange at 10% of the rate observed with saturating acetyl-CoA (McClure *et al.*, 1971c).

1.3.4 The Coupling of ATP Hydrolysis to Biotin Carboxylation

The free energy change ($\Delta G°'$) for hydrolysis of carboxybiotin has been calculated to be -4.7 kcal, and so carboxybiotin can be classified as a "high-energy" compound (Wood *et al.*, 1963). It is the hydrolysis of MgATP [$\Delta G°' = -7.6$ kcal (Wood *et al.*, 1966)] that drives the carboxylation of biotin. Three different mechanisms have been proposed for the coupling of these two reactions. They

are;

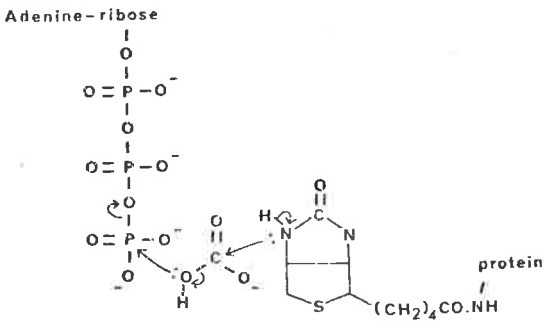
i) a concerted mechanism without the formation of a kinetically significant intermediate,

ii) enzyme activation, involving the formation of a phosphoryl or adenyl complex which then reacts with HCO_3^-,

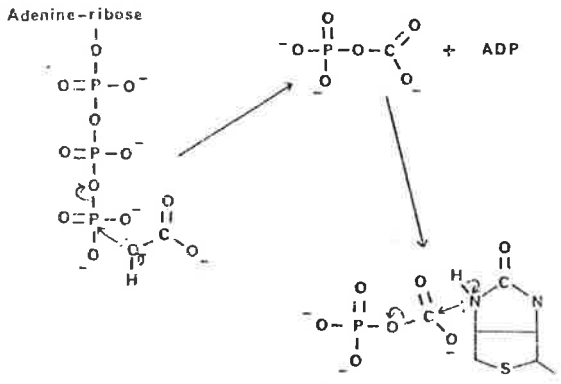
iii) bicarbonate activation with the formation of a phosphoryl or adenyl complex which then reacts with the biotin prosthetic group.

Enzyme activation by adenylation can be eliminated in the case of propionyl-CoA carboxylase on the basis of the work of Kaziro *et al.* (1962) and so is unlikely to apply to pyruvate carboxylase. Kaziro *et al.* found that when $HC^{18}O_3^-$ was used as a substrate, two ^{18}O atoms were found in the carboxyl group of methylmalonyl-CoA and one ^{18}O atom was incorporated into Pi. This is however, compatible with a concerted mechanism, and with a stepwise mechanism involving phosphorylation as can be seen from the example below.

(i) concerted mechanism



(ii) stepwise mechanism

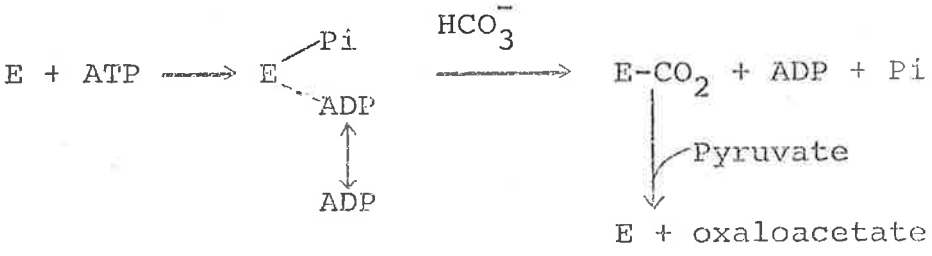


1.3.4.1 The concerted mechanism: In the concerted mechanism (shown above) proposed by Kaziro *et al.* (1962), HCO_3^- makes a nucleophilic attack on the γ-phosphate of ATP, in concert with a nucleophilic attack on the HCO_3^- by the 1'-N of biotin. The main evidence against this mechanism is the ability of pyruvate carboxylase to catalyse an ATP-ADP isotopic exchange reaction which does not require free Pi and does not involve biotin. When this exchange was detected in chicken liver by Scrutton & Utter (1965) they assumed it to result from an abortive side reaction, however recent experiments in this laboratory indicate that it is part of the main reaction pathway (N.B. Phillips, unpublished results).

1.3.4.2 The activated enzyme mechanism: This mechanism was proposed by Scrutton & Utter (1965) and is supported by the model studies of Kluger & Adawadkar (1976). However, if this mechanism applied one would expect the enzyme to catalyse an oxaloacetate-HCO_3^- isotopic exchange in the absence of ADP;

$$E\sim Pi + HCO_3^- \rightleftharpoons E_{Pi}^{CO_2} \overset{Pi}{\rightleftharpoons} E\sim CO_2 \overset{pyr}{\rightleftharpoons} E + \text{oxaloacetate}$$

Such an exchange cannot be found (Scutton & Utter, 1965; Ashman & Keech, 1975). To rationalize this observation, Scrutton & Utter proposed that the enzyme is activated by both MgADP and Pi;



If this were the case, one would expect there to be an isotopic exchange between HCO_3^- and oxaloacetate which does not require phosphorylation of MgADP. However, Ashman & Keech (1975) have shown that analogues of ADP which are not phosphorylated by the enzyme (adenosine 5'-phosphosulphate and α,β-methylene ADP) will not support an HCO_3^--oxaloacetate isotopic exchange reaction. There still remains the possibility that Pi and ADP both bind covalently to the enzyme, but this would be most unlikely.

1.3.4.3 Substrate activation: As the possibility of substrate activation by adenylation has been discounted (Section 1.3.4), only activation of HCO_3^- by phosphorylation remains to be considered. Several lines of evidence support this mechanism. Carboxyphosphate, the condensation product of bicarbonate and phosphate, is the likely candidate for a reaction intermediate. Polakis *et al.* (1972, 1974), using acetyl-CoA carboxylase and Ashman & Keech (1975), using pyruvate carboxylase have shown that MgATP is formed from MgADP and carbamyl phosphate, an analogue of the unstable carboxyphosphate. Polakis *et al.* also showed that biotin is not involved catalytically in the reaction, as analogues of biotin substituted at the 1'-N, and therefore incapable of being carboxylated, can support the reaction.

Phosphonacetic acid, on the other hand, is an analogue of carboxyphosphate which will not phosphorylate MgADP because the labile C-O-P linkage is replaced by a stable $C-CH_2-P$ linkage. This compound is an effective inhibitor (Ashman & Keech, 1975), again implicating a carboxyphosphate intermediate or transition-state complex.

1.3.5 Transcarboxylation

Transcarboxylation, the second partial reaction catalysed by pyruvate carboxylase, involves the transfer of a carboxyl group from the biotin prosthetic group to a keto-acid substrate:

ENZ-carboxybiotin + pyruvate $\rightleftharpoons$ ENZ-biotin + oxaloacetate.

Pyruvate can be replaced by 2-oxobutyrate, 2-ketovalerate, or fluoropyruvate, but with a lower Vmax (Keech & Utter, 1963; Cheung & Walsh, 1976).

Isotopic exchange between [^{14}C]pyruvate and oxaloacetate occurs at about half the rate of the overall reaction (Scrutton *et al.*, 1965; Ashman & Keech, 1975). No activators are required for this reaction, although acetyl-CoA enhances the rate of this exchange catalysed by the rat, sheep and chicken enzymes by 2- to 3-folds (McClure *et al.*, 1971c; Ashman & Keech, 1972; unpublished observations).

Rose (1970) studied the stereochemistry of the second partial reaction using prochiral [^{3}H]pyruvate and found firstly that the reaction has a primary isotope effect and secondly that the transfer of the carboxy group from carboxybiotin to pyruvate occurs with retention of configuration at C-3 of pyruvate. Rose suggested that this result might be interpreted in terms of a concerted mechanism, as proposed previously by Mildvan & Scrutton, (1967) from their NMR studies. Cheung & Walsh (1976) later used the kinetic isotope effect to demonstrate that the slowest step in the overall reaction was in the carboxylation of ENZ-biotin by ATP and HCO_3^-. In addition, their calculations indicated that once pyruvate bound to the enzyme it was

only 50% committed to catalysis; that is pyruvate dissociated from the enzyme without reacting one out of two times it bound. As discussed previously (Section 1.3.2.4) Easterbrook-Smith *et al.* (1976a) have shown that the binding of pyruvate can bring about decarboxylation of the ENZ-carboxybiotin without concomitant formation of oxaloacetate.

1.3.6 The Bound Metal Ion

All of the eukaryote pyruvate carboxylases studied so far have a tightly bound metal ion - either Mn^{2+}, Zn^{2+}, Co^{2+} or Mg^{2+}, depending on the species (for review see Scrutton & Young, 1972). The proton-NMR studies of Mildvan & Scrutton (1967) indicated that in chicken liver pyruvate carboxylase the bound Mn^{2+} is located at the transcarboxylation site. Mildvan & Scrutton suggested that pyruvate bound with monodentate co-ordination of its carbonyl group to the enzyme-bound Mn^{2+}, and was thereby electrophilically activated. However, the ^{13}C-NMR studies of Fung *et al.* (1973) and the proton-NMR and EPR studies of Reed & Scrutton (1974) show that pyruvate binds too far from the Mn^{2+} atom to be in the Mn^{2+} inner co-ordination sphere. These investigators suggested that the bound metal is buried deep in the protein and that there is a tightly co-ordinated water molecule between the metal and the pyruvate. According to this scheme the Mn^{2+} promotes the acidity of the bridging water molecule, which in turn promotes enolization of the pyruvate, facilitating carboxyl transfer to the pyruvate. However, the case for a catalytic role of the bound metal has not been proven.

1.3.7 Appendix

Derivation of rate equation for scheme depicted in Fig. 1.3.

Abbreviations:

A	[ATP]
OAA	[oxaloacetate]
P	[pyruvate]
E	[enzyme]
EA	[E.ATP]
EC	[E.CO_2]
ECP	[E.CO_2.pyruvate]
E..C	[E..CO_2] (labile carboxyenzyme)
E_T	[total enzyme]

As the rate is measured in terms of OAA production,

$$v = k_4 ECP$$

Under steady state conditions

$$E..C = \left[\frac{k_5}{k_{-5}P + k_6}\right] ECP$$

$$EC = \frac{1}{k_3 P}\left[k_{-3} + k_4 + \frac{k_5 k_6}{k_{-5}P + k_6}\right] ECP$$

$$EA = \left[k_4 + \frac{k_5 k_6}{k_{-5}P + k_6}\right] ECP$$

$$E = \left[\frac{k_{-1} + k_2}{k_1 A}\right]\left[k_4 + \frac{k_5 k_6}{k_{-5}P + k_6}\right] ECP$$

Now $ECP = E_T - (E + EA + EC + E..C)$

$= E_T - \Delta ECP$ where Δ is the sum of terms above

So $ECP = \frac{E_T}{\Delta + 1}$

$$\text{and} \quad v = \frac{k_4 \, E_T}{\Delta + 1}$$

$$\text{or} \quad \frac{E_T}{v} = \frac{\Delta + 1}{k_4}$$

$$= \frac{1}{A} \left[\frac{k_{-1} + k_2}{k_1} \right] \left[1 + \frac{k_5 \, k_6}{k_4 \, (k_{-5}P + k_6)} \right]$$

$$+ \frac{k_5}{k_4 \, (k_{-5}P + k_6)} \left[k_6 + \frac{k_6}{k_3 P} + 1 \right]$$

$$+ \frac{1}{k_3 P} \left[\frac{k_{-3}}{k_4} + 1 \right] + \frac{1}{k_4} + 1$$

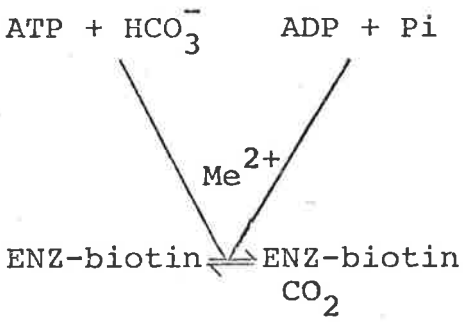
Thus in a reciprocal plot with varying ATP the slope will be

$$\left[\frac{k_{-1} + k_2}{k_1} \right] \left[1 + \frac{k_5 \, k_6}{k_4 \, (k_{-5}P + k_6)} \right]$$

TABLE 1.1

REACTIONS CATALYSED BY BIOTIN-DEPENDENT ENZYMES

A. Carboxylases



First partial reaction		Second partial reaction
ENZ-biotin-CO_2		ENZ-biotin
+ acetyl-CoA	$\overset{1}{\rightleftharpoons}$	+ malonyl-CoA
+ propionyl-CoA	$\overset{2}{\rightleftharpoons}$	+ methylmalonyl-CoA
+ β-methyl-crotonyl-CoA	$\overset{3}{\rightleftharpoons}$	+ β-methyl-glutaconyl-CoA
+ geranyl-CoA	$\overset{4}{\rightleftharpoons}$	+ carboxy geranyl-CoA
+ pyruvate	$\overset{5}{\rightleftharpoons}$	+ oxaloacetate
+ urea	$\overset{6}{\rightleftharpoons}$	+ N-carboxyurea

B. Decarboxylases

ENZ-biotin		ENZ-biotin-CO_2	ENZ-biotin CO_2		ENZ-biotin
+ methylmalonyl-CoA	$\overset{7}{\rightleftharpoons}$	+ propionyl-CoA	+ H_2O	$\rightleftharpoons$	+ HCO_3^-
+ oxaloacetate	$\overset{8}{\rightleftharpoons}$	+ pyruvate			

C. Transcarboxylase

Methylmalonyl-CoA + ENZ-biotin $\overset{9}{\rightleftharpoons}$ propionyl-CoA + ENZ-biotin-CO_2

ENZ-biotin-CO_2 + pyruvate $\overset{9}{\rightleftharpoons}$ ENZ-biotin + oxaloacetate

1 acetyl-CoA carboxylase
2 propionyl-CoA carboxylase
3 β-methylcrotonyl-CoA carboxylase
4 geranyl-CoA carboxylase
5 pyruvate carboxylase
6 urea carboxylase
7 methylmalonyl-CoA decarboxylase
8 oxaloacetate decarboxylase
9 transcarboxylase

TABLE 1.2

MOLECULAR WEIGHTS OF SOME BIOTIN-DEPENDENT ENZYMES

ENZYME	SOURCE	SUBUNIT M.W.s ($\times 10^{-3}$)		NATIVE M.W.	BIOTIN-CONTAINING SUBUNIT
Propionyl-CoA carboxylase					
	Mycobacterium smegmatis[1]	57	64	500	Large
	Bovine kidney[2]	58	74	-	Large
	Sheep liver[10]	58	72	-	Large
	Pig Heart[2]	55	78	700[3]	Large
	Human liver[6]	56	72	540	Large
Methylcrotonyl-CoA carboxylase					
	Bovine kidney[2]	62	80	-	Large
	Pseudomonas citronellolis[4]	63	73	520 - 580	Large
	Achromobacter IVS[5]	78	96	700	Large
Geranyl-CoA carboxylase					
	Pseudomonas critronellolis[4]	63	75	520 - 580	Large
"Acyl-CoA carboxylase"					
	Turbatrix aceti (nematode)[7]	58	82	667	Large
Pyruvate carboxylase					
	Pseudomonas citronellolis[8,9]	54	65	454 - 530	Large

1 Henrikson & Allen (1979)
2 Lau *et al.* (1979)
3 Kaziro *et al.* (1961)
4 Fall & Hector (1977)
5 Schiele *et al.* (1975)
6 Kalousek *et al.* (1980)
7 Meyer *et al.* (1978)
8 Barden *et al.* (1975)
9 Cohen *et al.* (1979)
10 See Chapter 3.

TABLE 1.3

INITIAL VELOCITY PATTERNS FOR VARIOUS PYRUVATE CARBOXYLASES

VARIABLE SUBSTRATE	CHANGING FIXED SUBSTRATE	OBSERVED PATTERNS[a] (1)	(2)	(3)	(4)	PYRUVATE CONCENTRATION (mM) (1)	(2)	(3)	(4)
MgATP	HCO_3^-		I	I	I		3	5,100	10
	pyruvate	I[b]	P	I	I[b]	0.04-4.4	$\leq$3	0.029-2.2	0.2-5
HCO_3^-	MgATP	I	I	I	I	5	3	5	10
	pyruvate	I[b]	P	I	I[b]	0.04-4.4	$\leq$3	?	0.1-10
Pyruvate	MgATP		P	I	I[c]		$\leq$3	?	0.2-5
	HCO_3^-		P	I	I[c]		$\leq$3	?	0.1-10

Sources:

1 rat liver (McClure *et al.*, 1971b)

2 chicken liver (Barden *et al.*, 1972)

3 pig liver (Warren & Tipton, 1974b)

4 Sheep kidney (Ashman & Keech, 1975)

a. I represents intersecting and P parallel lines

b. Slope replots were concave downwards

c. Double reciprocal plots were concave downwards.

TABLE 1.4

PRODUCT INHIBITION KINETIC PATTERNS

FOR VARIOUS PYRUVATE CARBOXYLASES

PRODUCT INHIBITOR	VARIED SUBSTRATE	INHIBITION PATTERN[a] (1)[b]	(2)[b]	(3)[b]	(4)[b]
MgADP	MgATP	C	C	C	C
	HCO_3^-	-	NC	M	NC
	pyruvate	UC	UC	M	UC
Pi	MgATP	C	C	C	C
	HCO_3^-	-	NC	M	NC
	pyruvate	UC	UC	M	UC, NC[d]
oxaloacetate	MgATP	-	NC	M[c]	NC
	HCO_3^-	-	NC	M	NC
	pyruvate	-	C	C	C

a. abbreviations

C - competitive; NC - non-competitive; UC - uncompetitive; M - mixed.

b. Sources:

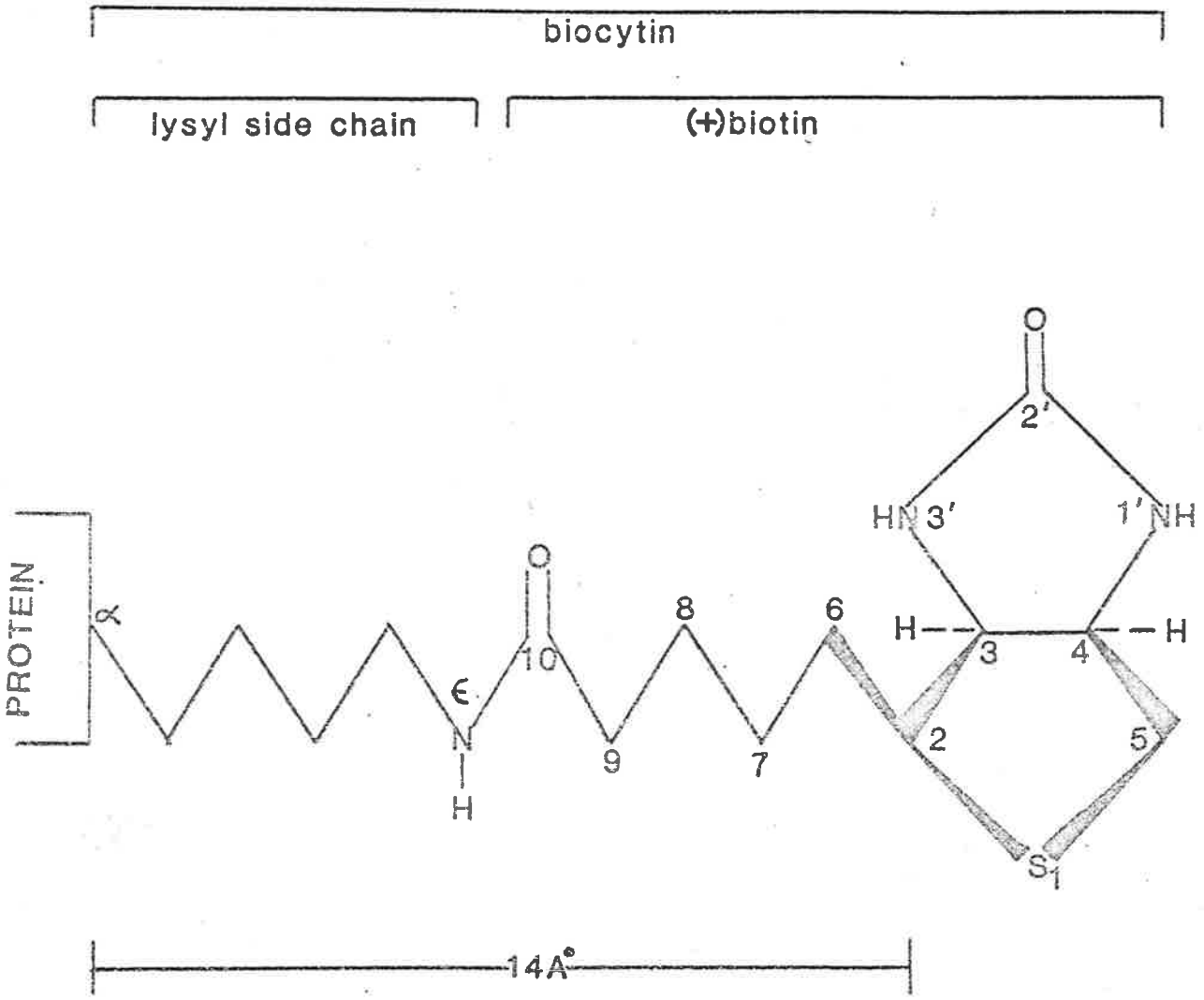
1. rat liver (McClure *et al.*, 1971b)
2. chicken liver (Barden *et al.*, 1972)
3. pig liver (Warren & Tipton, 1974b)
4. sheep kidney (Ashman & Keech, 1975)

c. Referred to as both mixed and non-competitive by Warren & Tipton.

d. At low and high pyruvate concentration, respectively.

FIGURE 1.1

BIOCYTIN



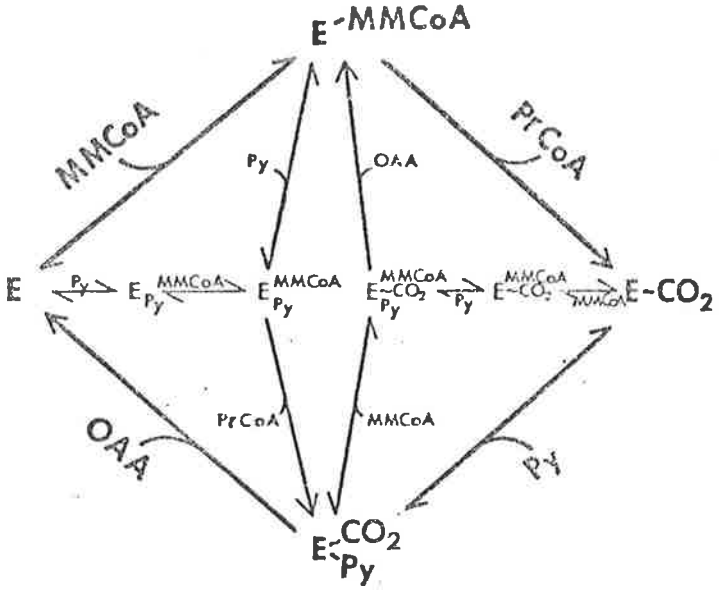


FIGURE 1.2

STEADY STATE MODEL OF THE TRANSCARBOXYLASE REACTION PROPOSED BY NORTHROP (1969)

The primary ping-pong sequence of the reaction is shown in boldface around the outside of the model. Alternative random pathways are shown near the centre. The abbreviations used are MMCoA, methylmalonyl-CoA; PrCoA, propionyl-CoA; Py, pyruvate; OAA, oxaloacetate.

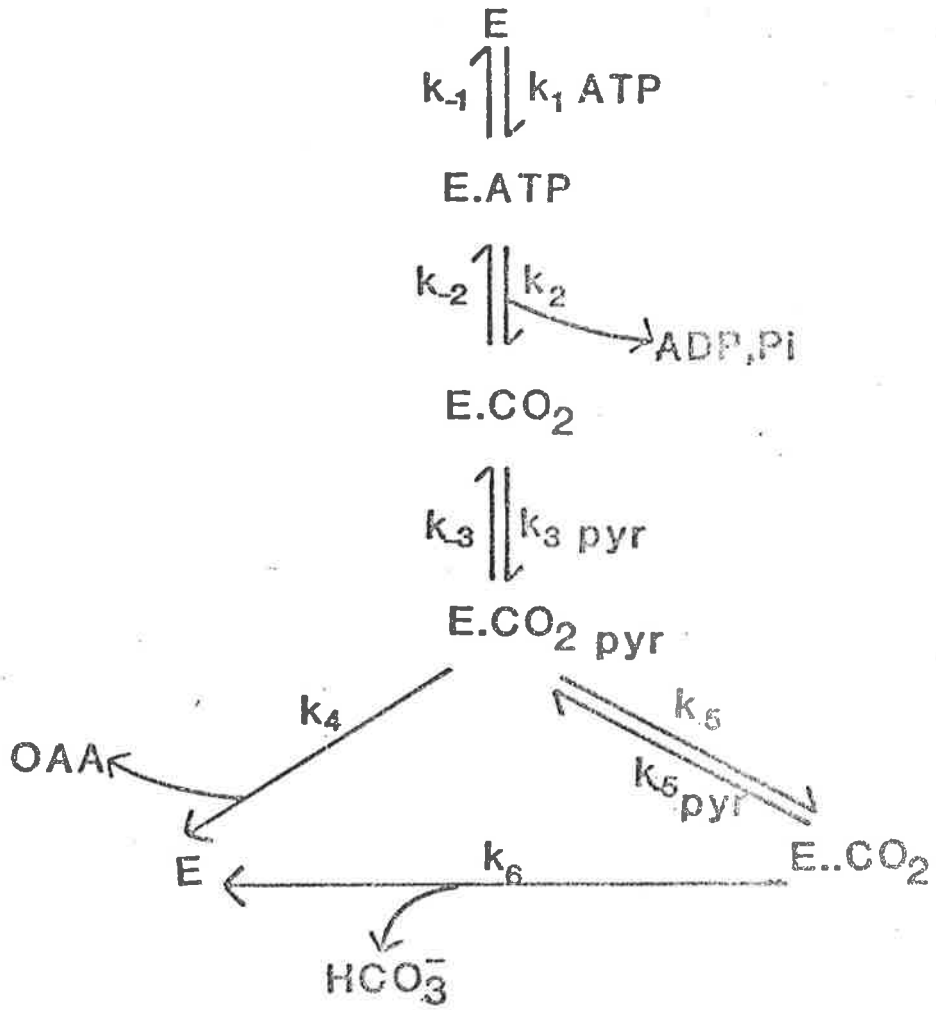


FIGURE 1.3

A ping-pong mechanism for pyruvate carboxylase which incorporates alternative pathways of enzyme decarboxylation. As HCO_3^- is assumed to be saturating its binding is not depicted.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Enzymes and Proteins

Albumin, bovine serum; β-galactosidase (E.C.2.3.1.23), grade IV from *E. coli*; malate dehydrogenase (E.C.1.1.1.37), glutamate:pyruvate transaminase (E.C.2.6.1.2), and glutamate: oxaloacetate transaminase (E.C.2.6.1.1) each from pig heart, 3'-nucleotidase (E.C.3.1.3.6), type III from rye grass; phosphorylase b (E.C.2.4.1.1) from rabbit muscle, were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Catalase (E.C.1.11.1.6) from bovine liver; aldolase (E.C. 4.1.2.7) from rabbit muscle; thyroglobulin from bovine thyroid were from Pharmacia, Uppsala, Sweden. Avidin from egg white and TPCK-treated trypsin (E.C.3.4.4.4) from bovine pancreas were from Worthington Biochemical Corp. Freehold, N.J., U.S.A. Pronase, (B grade) was obtained from Calbiochem. (Aust.) Pty. Ltd., Sydney, Australia. Ferritin (Cd-free) from horse spleen, was obtained from Boehringer Mannheim, GmbH, W. Germany.

Urease (E.C.3.5.1.5) from jack bean was the generous gift of Dr. R.L. Blakeley.

2.1.2 Radioactive Chemicals

[U-^{14}C]ATP, d[carbonyl-^{14}C]biotin, N-ethyl[2,3-^{14}C]-maleimide, $^{3}H_2O$, sodium [^{14}C]bicarbonate, and sodium [2-^{14}C]pyruvate were obtained from The Radiochemical Centre, Amersham, England. [G-^{3}H]Coenzyme A was obtained from New England Nuclear, Boston, Mass., U.S.A. [γ-^{32}P]ATP was a gift from Dr. R.H. Symons.

2.1.3 General Chemicals

ATP (disodium salt, grade I), CoA (grade I), NADH, 2-mercaptoethanol, DTE, fluoropyruvate, oxaloacetic acid, d-biotin, phospho(enol)pyruvate (monocyclohexylammonium salt), sodium 2-oxobutyrate, sodium glyoxylate monohydrate, sodium pyruvate (type II, dimer free), pyridoxal phosphate, sodium dodecylsulphate, Trizma base, N-ethylmorpholine, EDTA (disodium salt) and acrylamide were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sodium glutamate and semicarbazide HCl (AnalaR) were obtained from British Drug Houses, Poole, England. Coomassie brilliant blue was from Schwartz/Mann, Orangeburg, N.Y., U.S.A. Lithium hydroxypyruvate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. p-Toluenesulphonyl-N-methyl-N-nitrosamide was from TCI., Tokyo, Japan. Oxamic acid was from K. & K. Labs., Plainview, N.Y., U.S.A. $MgCl_2$ was prepared from spectroscopically pure Mg as described by Bais & Keech (1972) and standardized by atomic absorption spectrophotometry.

N,N-methylenebisacrylamide, N,N,N,N-tetramethylethylene-diamine, thin layer cellulose and silica gel plates were obtained from Eastman Kodak, Rochester, N.Y., U.S.A. Polyamide plates were from Chen-Ching Trading Co., Taipei, Taiwan. Polyethyleneimine thin layers were from Machery-Nagel & Co., Duren, Germany.

PPO (2,5-diphenyloxazole), POPOP (1,4-bis-2(4-methyl-5-phenoxazolyl)-benzene), and iodoacetic acid were supplied by Koch-Light Laboratories Ltd., Bucks, England. NCS tissue solubilizer was from Amersham/Searle Corp., Illinois, U.S.A.

Special enzyme grade ammonium sulphate was obtained from Mann Research Laboratories, N.Y., U.S.A. Polyethylene glycol (6000) was from Union Carbide Corporation. Sucrose, analytical reagent grade, was obtained from Colonial Sugar Refining Co., Syndey, Aust.

DEAE-Sephacel, phenyl-Sepharose and all grades of Sephadex, Sephacryl and Sepharose were obtained from Pharmacia, Uppsala, Sweden. DEAE-cellulose was obtained from Whatman Chromedia (W. & R. Balston, Ltd., England).

The protease inhibitor, "Trasylol" was obtained from Bayer Pharmaceutical Company., N.S.W., Aust.

2.2 METHODS

2.2.1 Preparation and Purification of Acetyl-CoA and Propionyl-CoA

Acetyl-CoA and propionyl-CoA were prepared from CoA and acetic anhydride or propionic anhydride by a slight modification to the method of Simon & Shemin (1953). The product was purified by ascending chromatography on acid-washed Whatman 3 MM paper using isobutyric acid-water-ammonia (66:33:1) as the developing solvent. The band was cut out and eluted with water. Only product with a 232/260 nm extinction ratio in the range 0.50 to 0.55 was used.

2.2.2 Protein estimation

Protein concentrations were determined by the spectrophotometric method described by Layne (1957), using the equation;

$$\text{protein concentration (mg ml}^{-1}) = 1.55\ A^{1\text{ cm}}_{280\text{ nm}} - 0.76\ A^{1\text{ cm}}_{260\text{ nm}}$$

2.2.3 Determination of Radioactivity

Samples dried on to solid supports (2 x 2 cm squares of Whatman 3 MM paper) were placed in vials containing 3 ml of toluene scintillation fluid [0.3% (w/v) 2,5-diphenyl-oxazole, 0.03% (w/v) 1,4-bis-2(4-methyl-5-phenoxazolyl)-benzene, in sulphur-free toluene (Bousquet & Christian, 1960)], and counted in a Packard Scintillation Spectrometer. Liquid samples were placed in vials containing a ten-fold volume excess of Triton X-100 scintillation fluid (toluene scintillation fluid, as above, and Triton X-100, 7:3 v/v), and counted in a Packard Scintillation Spectrometer. Polyacrylamide gel slices were immersed in 0.2 - 0.3 ml NCS solubilizer-water 9:1 (v/v) at 37°C for 4 h or until colourless, 3 ml of toluene scintillation fluid was added and the radioactivity counted.

2.2.4 Preparation of Sheep Liver Mitochondria

All procedures for the isolation of mitochondria were carried out at 4°C. Sheep livers were placed on ice as soon as possible after removal from the animal, and diced into small segments. These segments were homogenized in a Waring blender with 4 volumes (w/v) of 0.25 M sucrose containing 10^{-4} M EDTA. The homogenate was centrifuged at 600 g for 20 min to remove cell nuclei and whole cells, and the supernatant fraction centrifuged at 23,000 g for 20 min. The precipitated material was resuspended in 10^{-4} M EDTA and centrifuged again at 23,000 g for 20 min. The precipitate was suspended in a minimal volume of 10^{-4} M EDTA and freeze-dried. The dried mitochondria were stored in sealed plastic

bags at -15°C over silica gel.

2.2.5 Purification of Pyruvate Carboxylase

Pyruvate carboxylase was prepared from freeze-dried sheep and chicken liver mitochondria by the procedures described by Goss *et al.* (1979) except that a column of DEAE-Sepharose replaced the DEAE-Sephadex column. The sheep liver enzyme was used in all experiments except where stated otherwise.

2.2.6 Measurement of Propionyl-CoA Carboxylase Activity

In this procedure $H^{14}CO_3^-$ fixed in an acid stable form is measured, while unreacted $H^{14}CO_3^-$ is driven off by acidification and subsequent drying on paper squares. Assay solutions contained 0.2 M N-ethylmorpholine-acetate buffer, pH 8.0, 5 mM ATP, 25 mM $NaH^{14}CO_3$ (0.25 µCi/µmole), 1.5 mM propionyl-CoA, 10 mM $MgCl_2$, 100 mM KCl, 1 mM DTE and up to 0.05 units of propionyl-CoA carboxylase in a volume of 0.2 ml. The reaction was initiated by addition of enzyme and allowed to proceed for up to 5 min at 30°C before being quenched by the addition of 50 µl of 30% (w/v) trichloroacetic acid. Protein was sedimented by centrifuging for 1 min in an Eppendorf centrifuge and a 50 µl sample was applied to a 2 x 2 cm square of Whatman 3 MM paper. This was dried for 5 min at 100°C and the remaining radioactivity determined as described in Section 2.2.3. One unit of enzyme catalyses the formation of 1 µmole of methylmalonyl-CoA per min at 30°C.

2.2.7 Measurement of Pyruvate Carboxylase Activity

a). The spectrophotometric assay system: This continuous assay procedure, based on that described by Utter & Keech (1963) involves reduction of the oxaloacetate produced by the pyruvate carboxylase reaction, using malate dehydrogenase, with concomitant oxidation of NADH to NAD^+. Assay solutions contained 100 mM Tris-Cl, pH 8.4, 2.5 mM ATP, 5 mM $MgCl_2$, 20 mM $NaHCO_3$, 10 mM pyruvate (sodium salt), 0.25 mM acetyl-CoA, 0.125 mM NADH, 5 units of malate dehydrogenase and up to 0.1 units of pyruvate carboxylase in a volume of 1 ml.

The reaction was monitored at 340 nm using a Varian-Techtron 635-D spectrophotometer with a cell block thermostatted at 30°C. The rate of oxaloacetate synthesis was calculated using an extinction coefficient at 340 nm for NADH of 6.22 mM^{-1} cm^{-1} (Dawson *et al.*, 1969). One unit of enzyme catalyses the formation of 1 µmole of oxaloacetate per min at 30°C.

b). The radiochemical assay system: Assay solutions contained 100 mM Tris-Cl, pH 8.4, 2.5 mM ATP, 5 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (0.25 µCi/µmole). 10 mM sodium pyruvate, 0.25 mM acetyl-CoA and up to 0.06 units of pyruvate carboxylase, in a volume of 0.5 ml. The reaction was initiated by the addition of enzyme and allowed to proceed for up to 5 min at 30°C before being quenched by the addition of 50 µl of 2 M semicarbazide HCl. As well as terminating the reaction, this reagent drives off unreacted $H^{14}CO_3^-$ and stabilizes the oxaloacetate formed as its osazone derivative.

Protein was sedimented by centrifuging for 1 min in an Eppendorf centrifuge and samples of 50 µl applied to 2 x 2 cm squares of Whatman 3 MM paper, dried at 100°C for 5 min, and the remaining radioactivity determined as described in Section 2.2.3.

2.2.8 SDS-polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed using the discontinuous system of Laemmli (1970). Samples were dissolved in loading buffer, which contained 0.0625 M Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.002% bromphenol blue, and heated at 90°C for 5 min before loading on the gel. After electrophoresis the gel was stained by shaking in methanol-acetic acid-water (50:10:40) containing 0.1% Coomassie Brilliant Blue R250 and destained by shaking in methanol-acetic acid-water (5:10:85).

CHAPTER 3

PURIFICATION AND PROPERTIES OF PROPIONYL-CoA CARBOXYLASE

3.1 INTRODUCTION

Whether or not the biotin-dependent enzymes are related in an evolutionary sense, they are related to the extent that they carry out similar reactions. However, to date, there is insufficient information to draw general conclusions about the catalytic mechanisms of these enzymes. Some comparative studies of the pyruvate carboxylase and propionyl-CoA carboxylase reactions are described in Chapter 6.

Structural studies of biotin-dependent enzymes are at various stages of refinement and some similarities have been pointed out in Chapter 1. In this chapter I describe experiments on the purification and structure of propionyl-CoA carboxylase from sheep liver. This source was chosen, firstly, because the enzyme is quite abundant in sheep liver, as one might expect of an animal that relies primarily on propionate as a carbon source for gluconeogenesis (Jarrett & Ballard, 1976). Secondly, the enzyme can be obtained in a partially purified form (specific activity about 0.1) from material normally discarded during the purification of pyruvate carboxylase, which is prepared routinely in this laboratory.

3.2 METHODS

3.2.1 S-Carboxymethylation of Cysteinyl Residues in Polypeptides

The procedure is described for 10 mg of protein but can be scaled up for larger amounts. The protein was dissolved in 1 ml of 0.25 M Tris-Cl pH 8.0, 2 mM DTE along

with 573 mg of recrystallised guanidine hydrochloride. The tube was flushed with nitrogen and incubated at 37°C for 30 min, after which time, 15 µl of 1 M iodoacetic acid in 3 M Tris-Cl, pH 8.0, was added. The tube was again flushed with nitrogen and then incubated in the dark at 37°C for 1 h. At this stage a small aliquot of the solution was tested for the presence of free sulphydryl groups by reacting with dithiobis(2-nitrobenzoic acid). If the test was positive, the solution was incubated a further 30 min or until free thiol groups could not be detected. When the test was negative, unreacted iodoacetic acid was quenched by adding 1 ml of 20 mM DTE and the solution dialysed extensively against either water or 0.1 M ammonium bicarbonate.

3.2.2 Amino Acid Analysis

Samples were hydrolysed under nitrogen in sealed tubes with 1 ml of 6 M HCl containing one drop of 5% phenol (v/v). After hydrolysis at 110°C for the specified time, the hydrolysates were evaporated to dryness and analysed by the procedure of Piez & Morris (1960) using a Beckman 120C analyser modified as described by Harding & Rogers (1971).

3.2.3 Purification of the Subunits of Propionyl-CoA Carboxylase

About 2 mg of propionyl-CoA carboxylase was converted to the S-carboxymethyl form as described in Section 3.2.1 and subjected to preparative SDS-polyacrylamide gel electrophoresis in a 7.5% polyacrylamide gel. The bands were visualized by soaking the gel in 4 M sodium acetate (Higgins

& Dahmus, 1979). The protein was electrophoretically eluted, in the apparatus described by Symons (1978), with 0.07 M Tris-Cl and 0.05% SDS, pH 8.3 for 16 h and then for a further 8 h in the absence of SDS. The buffer was removed by dialysis against water and the samples freeze-dried.

3.2.4 Preparation of Apo-ferritin

Horse spleen ferritin (1 mg) was dialysed at 4°C for 18 h against 20 mM sodium acetate containing 2 mM EDTA and 10 mg of phenanthroline at pH 4.6 and then for 8 h against 50 mM N-ethylmorpholine-acetate, 2 mM EDTA and 1 mM glutathione, pH 7.5.

3.2.5 β-galactosidase Assay

The assay solution contained β-galactosidase, 0.55 mg/ml o-nitrophenyl-β-D-galactopyranoside, 1 mM DTE and 0.1 M potassium phosphate, pH 7.2. The absorbance change at 420 nm was monitored using a Varian 635-D spectrophotometer with thermostatted cuvette holders.

3.2.6 Electron Microscopy

Samples of enzyme containing 0.05 mg/ml protein were prepared for electron microscopy by a negative staining technique based on the procedure reported by Valentine *et al.* (1966) but modified by using 4% (w/v) uranyl acetate dissolved in water (pH 5.0) as negative staining solution (Gottschalk *et al.*, 1976). The protein was either unfixed, or crosslinked with dimethyl suberimidate (Davies & Stark, 1970). Samples were observed and micrographs taken with a Siemens 102 electron microscope operating at 60 or 80 kV accelerating voltage and at primary magnifications ranging

from 30,000 X to 80,000 X.

3.2.7 Gel Electrophoresis of Native Enzyme

A Pharmacia PAA4/30 4 - 30% polyacrylamide gradient gel was pre-electrophoresed for 20 min at 70 V with electrophoresis buffer, which consisted of 0.09 M Tris, 0.08 M boric acid and 2.5 mM EDTA, pH 8.4. Protein samples (2.5 - 5 µg) were dissolved in electrophoresis buffer containing 10% glycerol and 0.1% bromphenol blue and then loaded onto the gel. After an initial 15 - 20 min at 70 V, the samples were electrophoresed for at least 2,000 V-hours.

3.2.8 Sephacryl S-300 Gel Chromatography

Samples were loaded on a 50 x 1.6 cm column of Sephacryl S-300 which had been equilibrated with 0.05 M N-ethylmorpholine buffer, pH 7.5, containing 1 mM EDTA and 0.5 mM DTE. The proteins were eluted with the same buffer at a flow rate of 0.192 ml per min, collecting fractions of 1.28 ml.

3.3 PURIFICATION

3.3.1 Initial Extraction and Ammonium Sulphate Fractionation

Propionyl-CoA carboxylase was purified by suspending 120 g of freeze-dried sheep liver mitochondria in 1750 ml of extraction buffer which contained 25 mM Tris-acetate, pH 6.7, 3.5 mM $MgCl_2$ and 1.7 mM ATP. The pH was maintained between 6.5 and 6.7 during addition of the mitochondria. The suspension was stirred for 20 min and undissolved material removed by centrifuging at 23,000 g for 10 min

at 4°C. Ammonium sulphate was added to the supernatant to a final concentration of 1.37 M while maintaining the pH between 7.0 and 7.2. The suspension was stirred for 20 min and precipitated protein removed by centrifuging at 23,000 g for 20 min at 4°C. At this stage the precipitated material contains pyruvate carboxylase while propionyl-CoA carboxylase remains in the supernatant. Ammonium sulphate and DTE were added to the supernatant to final concentrations of 2.46 and 0.5 mM, respectively. The pH was adjusted to 7.5 while the suspension was stirred for 20 min, after which the precipitated protein was collected by centrifuging at 23,000 g for 20 min at 4°C. At this stage the precipitated enzyme can be stored at 4°C for a few days or at -15°C for several weeks without loss of activity.

3.3.2 Polyethylene Glycol Fractionation

Polyethylene glycol fractionation gives a small increase in specific activity and desalts the solution prior to ion exchange chromatography. The precipitate from the previous ammonium sulphate fractionation was dissolved in 250 ml of buffer A, containing 0.02 M N-ethylmorpholine-acetate, pH 7.5, 1 mM EDTA and 0.5 mM DTE. Polyethylene glycol (6000) was added in the ratio of 17 g per 100 ml of solution. The suspension was stirred for 20 min after all the polyethylene glycol had dissolved and then centrifuged at 18,000 g for 15 min at 4°C. Centrifugation at higher speeds gave a pellet that was difficult to dissolve. The precipitate was dissolved in 250 ml of buffer A and centrifuged at 23,000 g for 15 min

to remove any undissolved material.

3.3.3 Ion Exchange Chromatography On DEAE-Sephacel

The supernatant solution from the previous step was added to a DEAE-Sephacel column (5 x 30 cm), previously equilibrated with buffer B, containing 0.05 M N-ethyl-morpholine-acetate, pH 7.5, 1 mM EDTA and 0.5 mM DTE. The column was washed with a linear gradient of 0 to 0.05 M KCl in one litre of buffer B and the enzyme eluted with a two litre gradient of 0.05 to 0.2 M KCl in buffer B, collecting the eluate in 15 ml fractions. The fractions were assayed for protein concentration and enzymic activity and the fractions with the highest specific activity pooled. SDS-gel electrophoresis of the pooled fractions revealed six major and a few minor polypeptides (Fig. 3.1).

3.3.4 Hydrophobic Chromatography

Ammonium sulphate (2.8 g per 100 ml) was added to the pooled fractions from the DEAE-Sephacel column to enhance hydrophobic interaction. The solution was loaded on a phenyl-Sepharose column (2.5 x 20 cm) previously equilibrated with buffer B plus 0.41 M ammonium sulphate. The enzyme was eluted with a negative linear gradient starting with 0.2 M ammonium sulphate in buffer B and ending with buffer A, the total gradient volume being 800 ml. Fractions were assayed for protein concentration and enzymic activity and the fractions of highest specific activity pooled. The enzyme was concentrated by precipitation with 2.46 M ammonium sulphate and centrifuged at 23,000 g for 20 min. The pellet was dissolved in a minimum volume of 0.02 M N-ethylmorpholine-

acetate, pH 7.5, containing 1 mM EDTA and 1 mM DTE, and stored at -15°C. Under these conditions the enzyme loses 20 - 40% of its activity over 12 months. Enzyme purified to this stage was frequently homogeneous, as judged by SDS-polyacrylamide gel electrophoresis, although faint contaminating bands were occasionally observed (Fig. 3.1). The specific activity was in the range 15 - 27 units/mg. A summary of the purification steps and yields obtained is shown in Table 3.1.

3.3.5 Discussion

The purification procedure I have described compares favourably with the various procedures published recently for propionyl-CoA carboxylases from different sources (Meyer *et al.*, 1978; Lau *et al.*, 1979; Henrikson & Allen, 1979; Kalousek *et al.*, 1980; Gravel *et al.*, 1980). It is interesting that Henrikson & Allen and Gravel *et al.* have successfully used a monomeric avidin affinity column. The high affinity of native avidin for biotin [$K_D = 10^{-15}$ M, (Green, 1963)] has hindered the development of a suitable avidin affinity column for purifying native biotin-containing enzymes. Use of an avidin monomer affinity column is a possible alternative to the hydrophobic chromatography employed in this study. This may give higher yields than the 50 - 70% recovery obtained from the hydrophobic column. This advantage may be offset, however, by the need to concentrate or desalt the enzyme before loading on an avidin affinity column. (Henrikson & Allen lose 25% in doing this.). Hydrophobic chromatography gave better results

than chromatography on agarose-hexane-CoA, blue dextran-Sepharose or biotin-Sepharose.

3.4 CRYSTALLIZATION

Propionyl-CoA carboxylase is a large protein and the determination of its three-dimensional structure by X-ray crystallography may seem a daunting project. However, the structural information obtained by this procedure is unsurpassed and is essential for a detailed understanding of the catalytic mechanism. Although the crystals obtained in this study were not large enough for a thorough X-ray diffraction study, the diffraction pattern obtained indicates that they are of sufficient quality to warrant further attempts (P.F. Colman, personal communication). Therefore, I describe here the crystallization techniques I have applied, successful and unsuccessful.

3.4.1 Crystallization Method and Description of Crystals

A solution of propionyl-CoA carboxylase (2 mg/ml, specific activity 24) in 2 ml of 0.05 M N-ethylmorpholine-acetate, pH 7.5, with 1 mM DTE and 1 mM EDTA was gently stirred at room temperature in a small glass bottle. A saturated solution of ammonium sulphate pH 7.2, was added dropwise until the solution became quite turbid. A small volume of water was added so that the solution appeared only slightly turbid. The bottle was then stored at 4°C, covered by an inverted beaker, so that slow evaporation of the liquid could occur. After several weeks a sample of the suspension was diluted into a 50% saturated solution of ammonium

sulphate pH 7.2, and examined with a dissecting microscope with polarizing filters attached. Many crystals could be seen, as well as amorphous clumps of precipitated protein. The crystals were hexagonal bipyramidal in shape and generally uniform in size, with a diameter of approximately 0.1 mm (Fig. 3.2).

Several crystals were picked out of the mother liquor with capillary tubes and washed thoroughly in 50% saturated ammonium sulphate solution before being assayed for enzymic activity. The crystals dissolved in assay solution and still retained enzymic activity.

3.4.2 Vapour Diffusion Method

The vapour diffusion method was used in an attempt to grow larger crystals. In this procedure, enzyme in a small volume of ammonium sulphate solution is placed in an air-tight container along with a reservoir of ammonium sulphate solution at a different concentration. A slow equilibration of vapour pressures occurs, the rate and endpoint of which depends on the relative volumes and salt concentrations of the two solutions. In all of the conditions used the enzyme failed to crystallize. These conditions are summarized in Table 3.2.

3.5 PROPERTIES OF PROPIONYL-CoA CARBOXYLASE

3.5.1 Amino Acid Composition of the Native Enzyme

Amino acid analyses were performed on each of three high purity preparations (as judged by SDS-polyacrylamide gel electrophoresis) of propionyl-CoA carboxylase. Acid

hydrolysis was performed for 24, 48 and 72 h and in the case of threonine and serine the measurements were extrapolated to zero hydrolysis time. The results of the analyses are shown in Table 3.3. A comparison of the amino acid composition of propionyl-CoA carboxylase with a reference set of 207 mutually nonhomologous proteins (Reeck & Fisher, 1973) is shown in Table 3.4. The composition is not particularly unusual, with all except two amino acids falling within one standard deviation of the population mean of the reference set. Phenylalanine and arginine are present in slightly higher than usual proportions. When the basic (lys + arg + his), aromatic (phe + tyr) or hydrophobic (leu + ile + val + met + phe) residues are grouped, propionyl-CoA carboxylase has slightly more basic and aromatic residues and slightly fewer hydrophobic residues than the average (Table 3.5).

3.5.2 Amino Acid Compositions of the Subunits

The subunits were separated electrophoretically, as described in the Methods chapter. After 21 h hydrolysis, each subunit was subjected to amino acid analysis. The amino acid compositions are shown in Table 3.6. Comparisons with the compositions of other biotin-dependent enzymes are described in Chapter 5.

3.5.3 Subunit Molecular Weights

SDS-polyacrylamide gel electrophoresis of propionyl-CoA carboxylase reveals that it is comprised of two different subunits (Fig. 3.1). It is unlikely that the two polypeptides are the products of a cleavage by protease of a

single large polypeptide, as enzyme purified in the presence of the protease inhibitor Trasylol (≥ 100 units/ml) has the same appearance on SDS-polyacrylamide gels. Densitometric scans of stained gels indicate the molar ratio of the two bands is very close to unity.

The molecular weights of the subunits were measured by electrophoresis in 10% polyacrylamide-SDS, along with markers (Table 3.7). To accurately determine the relative mobilities of the proteins, a track which contained all of the markers and also propionyl-CoA carboxylase was cut out of the slab gel and scanned at 515 nm using a Varian spectrophotometer with a gel scanner attachment. The molecular weights and relative mobilities of the proteins are shown in Figure 3.3. The estimated molecular weights of the two subunits of propionyl-CoA carboxylase are 58,000 and 72,000.

3.5.4 Localization of the Biotin Prosthetic Group

The biotin-containing subunit was identified both by biotin assay of isolated subunits and by SDS-polyacrylamide gel electrophoresis of avidin-treated enzyme (Lau *et al.*, 1979). In the first method the subunits were separated as described in the methods section and individually hydrolysed for 21 h. The biotin content of each was determined by the method of Rylatt *et al.* (1977). The 58,000 dalton subunit contained no biotin while the 72,000 dalton subunit contained 2.9 nmol of biotin per 0.47 mg of polypeptide (measured by amino acid analysis) which corresponds to 0.44 moles of biotin per mole of subunit. The low ratio of biotin to subunit is due to loss of biotin during acid

hydrolysis.

The above findings were confirmed in the following manner. Propionyl-CoA carboxylase (5 µg) was mixed with 5 µg of avidin in 10 µl of 20 mM N-ethylmorpholine, pH 7.5, for 10 min at room temperature. This solution was then prepared for SDS-electrophoresis, omitting the usual heating step. A sample of propionyl-CoA carboxylase with no added avidin was prepared in the same way. These were then subjected to electrophoresis in a 10% polyacrylamide-SDS slab gel. The avidin, by complexing with biotin, decreased the mobility of the larger subunit, while the mobility of the smaller subunit was unchanged (Fig. 3.4). Furthermore, nearly all of the avidin treated 72,000 dalton subunit had decreased mobility. Thus biotin is attached to the 72,000 dalton subunit but not the 58,000 dalton subunit, and nearly all of the 72,000 dalton subunits have biotin attached.

3.5.5 Electron Microscopy

Electron microscope studies were undertaken in an effort to elucidate the quaternary structure of propionyl-CoA carboxylase. Although many high resolution images were obtained, they were very complex and bore no resemblance to any of the known protein subunit arrangements (Fig. 3.5). It is possible the molecules were damaged and flattened by the staining procedure, but this is unlikely as similar projections were observed when the sample was prepared from a protein solution containing one or more substrates and also when the enzyme was crosslinked with dimethylsuberimidate

before staining. Furthermore, the apparent diameters of the molecules were 130 - 160 Å, the size expected of a protein with molecular weight of about 500,000. For example, pyruvate carboxylase (480,000 daltons) and glutamate dehydrogenase (300,000 daltons) have apparent diameters of 155 - 165 Å and 110 - 120 Å respectively when prepared for electron microscopy by the same method as was used here for propionyl-CoA carboxylase (Mayer *et al.*, 1980).

Samples of propionyl-CoA carboxylase prepared for electron microscopy invariably contained aggregated molecules. The size of the aggregates ranged from short chains of a few molecules to large clumps of hundreds of molecules. Some examples are shown in Fig. 3.6.

3.5.6 Polyacrylamide Gel Electrophoresis of Native Enzyme

When electrophoresed under non-denaturing conditions in a 4 - 30% polyacrylamide gradient gel with thyroglobulin and ferritin as markers, propionyl-CoA carboxylase had a mobility which corresponded to a protein of 840,000 daltons (Fig. 3.7). Electrophoresis for longer time periods gave similar results.

3.5.7 Gel Exclusion Chromatography

The elution of propionyl-CoA carboxylase from Sephacryl S-300 was compared with that of thyroglobulin and ferritin. The propionyl-CoA carboxylase was loaded on the column at a concentration of 50 µg/ml to minimize polymerization. It eluted in the same position as thyroglobulin which has

a molecular weight of 669,000 (Tarutani & Nobuo, 1969) (Fig. 3.8).

3.5.8 Sucrose Density Gradient Centrifugation

Propionyl-CoA carboxylase was sedimented in sucrose density gradients under various conditions. There were usually two peaks of enzymic activity in the sedimentation profile (Fig. 3.9). The major peak always sedimented slightly faster than thyroglobulin (668,000 daltons) and the minor peak slightly faster than the thyroglobulin dimer. Although some treatments affected the relative sizes of the two peaks, their sedimentation co-efficients were not much affected and the trough between the peaks did not return completely to the baseline. A typical calibration curve is shown in Figure 3.10. In this experiment the apparent molecular weight of propionyl-CoA carboxylase was 710,000.

In a different experiment a freshly prepared crude mitochondrial extract was sedimented in a sucrose density gradient. In this case only a single peak appeared in the activity profile of the gradient. The apparent molecular weight in this case was 810,000 (Fig. 3.11).

3.5.9 Discussion

Sheep liver propionyl-CoA carboxylase contains non-identical subunits - a larger, biotin-counting subunit of molecular weight 72,000 and a smaller, non-biotin-containing subunit with a molecular weight of 58,000. With the recent publication of the subunit molecular weights of various other biotin-dependent enzymes (see Table 1.2) it has become apparent that this subunit arrangement is similar to that of several of the other biotin enzymes,

namely 3-methylcrotonyl-CoA carboxylase, geranyl-CoA carboxylase and pyruvate carboxylase from *Pseudomonas citronellolis*. Such similarities are to be expected if, as has been suggested by Lynen (Lynen, 1975; Obermayer & Lynen, 1976) the genes for at least some of the biotin-containing enzymes are derived from a common ancestor. A further examination of the relatedness of the biotin-dependent enzymes was undertaken by comparing amino acid compositions, and the results of this comparison are presented in Chapter 5.

Investigations of the molecular weight of sheep liver propionyl-CoA carboxylase by a range of techniques returned values of between 670,000 and 840,000. While this is in agreement with the molecular weight reported for the enzyme from pig heart (Kaziro *et al.*, 1961), several lines of evidence indicate that the values obtained are apparent molecular weights of a self-associating enzyme. When the enzyme is sedimented in a sucrose density gradient, more than one peak of activity is frequently found. When samples from each peak are examined by electron microscopy, a high proportion of paired particles can be seen in the sample from the faster sedimenting peak, while a high proportion of single particles can be seen in the sample from the slower sedimenting peak. In both samples, a range of higher order aggregates can also been seen, but most of the particles are present as monomers and dimers. The higher order aggregates probably formed in the time during which the gradients were fractionated and the enzyme located. The fact that the slower sedimenting peak was quite sharp

and well separated from the faster peak indicates that the aggregation-disaggregation process is slow under the conditions used for centrifugation. As a significant number of aggregates are formed during the fractionation process (whether at 4°C or room temperature) the relative slowness of aggregation during centrifugation might be due to high pressures being unfavourable to a step in the self-association process with a positive volume change. Further electron microscope studies with various preparations of the enzyme invariably revealed a range of aggregate sizes. Of the various treatments applied, such as different buffers, high and low salt, urea and Triton X-100, none were capable of preventing aggregation. The aggregates were also present when phosphotungstic acid was used as a stain in preparing samples for electron microscopy.

Given that sheep liver propionyl-CoA carboxylase is a self-associating enzyme, the apparent molecular weights observed are probably greater than that of the single molecule. By analogy with other proteins, propionyl-CoA carboxylase probably has an $\alpha_4\beta_4$ subunit composition and thus a molecular weight of about 520,000. The enzyme has recently been purified from human liver, where it has sub-units of 72,000 and 56,000 daltons and a molecular weight of about 540,000 (Kalousek *et al.*, 1980). Aggregation was not reported.

TABLE 3.1

PURIFICATION OF PROPIONYL-CoA CARBOXYLASE

PROCEDURE	VOLUME (ml)	TOTAL ACTIVITY (units)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (units/ mg)	YIELD (%)
first ammonium sulphate supernatant	1360	1046	-	-	100
second ammonium sulphate precipitate	467	1030	11,440	0.09	98
17% polyethylene glycol	210	718	5,130	0.14	67
DEAE-Sephacel chromatography	300	756	296	2.55	72
hydrophobic chromatography	200	584	24.4	23.9	56

TABLE 3.2

CONDITIONS IN WHICH PROPIONYL-CoA CARBOXYLASE DOES NOT CRYSTALLIZE

	ENZYME SOLUTION		RESERVOIR SOLUTION	
	ENZYME (mg/ml)	AMMONIUM SULPHATE (% saturation)		AMMONIUM SULPHATE (% saturation)
A.	3.4	50	C.	60
	4.0	40		60
	4.7	30		60
	5.4	20		60
	5.9	12		45, 50, 55
	5.3	21		45, 50, 55
	4.8	29		45, 50, 55
B.	4.0	40	D.	45
	4.0	40		47.5
	4.0	40		50
	4.0	40		52.5
	4.0	40		55

A. Propionyl-CoA carboxylase, specific activity 10.6 in 20 mM N-ethylmorpholine-HCl, pH 7.2, 1 mM DTE. Volume 20 µl in perspex at room temperature.

B. Propionyl-CoA carboxylase, specific activity 17.8 in 20 mM N-ethylmorpholine-acetate, pH 7.5, 1 mM DTE. Volume 100 µl in glass at 4°C.

C. Ammonium sulphate in 2 ml of 0.2 M N-ethylmorpholine-acetate, pH 7.4.

D. Ammonium sulphate in 1 ml of water, pH 7.2.

TABLE 3.3

AMINO ACID ANALYSIS OF NATIVE PROPIONYL-CoA CARBOXYLASE

RESIDUE	HYDROLYSIS TIME[a] (HRS) 24	48	72	MEAN	STANDARD DEVIATION
	nmol/130,000 ng[c]				
lys	68	68	70	69	1.2
his	38	36	37	37	2.9
arg	74	76	78	76	2.5
asx	101	95	95	96	3.0
thr	60	59	59	61[b]	2.7
ser	72	69	67	74[b]	1.9
glx	101	99	99	99	2.3
pro	58	53	48	52	5.0
gly	93	92	92	92	1.5
ala	89	93	92	92	4.2
cys	23	23	22	23	1.1
val	81	84	82	82	5.0
met	35	35	36	35	1.5
ile	70	70	74	72	2.7
leu	81	84	82	82	3.0
tyr	45	44	45	45	1.5
phe	59	61	61	60	1.3

a. Three samples of propionyl-CoA carboxylase were each hydrolysed for the indicated times and the subsequent analyses averaged.

b. Extrapolated to zero hydrolysis time.

c. The molecular weight of an α β subunit pair is 130,000 (see Section 3.5.3).

TABLE 3.4

COMPARISON OF THE AMINO ACID COMPOSITION OF PROPIONYL-CoA CARBOXYLASE WITH A REFERENCE SET OF PROTEINS

AMINO ACID RESIDUE	PROPIONYL-CoA CARBOXYLASE MOLE %	S.D.	REFERENCE SET MEAN MOLE %	S.D.
lys	5.9	0.1	6.5	2.7
his	3.2	0.2	2.2	1.2
arg	6.5	0.2	4.4	2.0
asx	8.2	0.3	10.6	2.6
thr	5.2	0.2	5.7	1.9
ser	6.3	0.2	6.6	4.8
glx	8.5	0.2	10.5	3.4
pro	5.3	0.3	4.8	2.1
gly	7.9	0.1	8.1	3.1
ala	7.8	0.4	8.4	2.8
cys	2.0	0.1	2.3	2.7
val	7.0	0.4	6.7	2.0
met	3.0	0.1	1.9	1.1
ile	6.1	0.2	4.9	1.8
leu	7.1	0.3	8.1	2.5
tyr	3.8	0.1	3.2	1.6
phe	5.2	0.1	3.7	1.4

The amino acid composition of propionyl-CoA carboxylase shown in Table 3.3 is expressed here in mole %. The reference set of 207 non-homologous proteins is taken from Reeck & Fisher (1973). The standard deviations in the reference set represent the variations among different proteins while the standard deviations in the propionyl CoA carboxylase composition represent errors in estimation.

TABLE 3.5

COMPARISON OF THE COMPOSITION OF PROPIONYL-CoA CARBOXYLASE WITH A REFERENCE SET OF PROTEINS

TYPE OF RESIDUE	PROPIONYL-CoA CARBOXYLASE %	REFERENCE SET %	S.D.
basic (K + R + H)	15.7	13.1	3.6
aromatic (F + Y)	9.1	6.9	2.1
hydrophobic (I + L + V + F + M)	23.9	25.3	4.1

The data are taken from Table 3.4 and grouped according to residue type.

TABLE 3.6

AMINO ACID COMPOSITION OF THE SUBUNITS OF PROPIONYL-CoA CARBOXYLASE

AMINO ACID	58,000 SUBUNIT	72,000 SUBUNIT (mole %)	NATIVE ENZYME
lys	5.1	6.5	5.9
his	3.7	3.9	3.2
arg	5.4	6.0	6.5
asx	11.2	9.2	8.2
thr	5.9	5.9	5.2
ser	6.4	7.3	6.3
glx	7.9	8.6	8.5
pro	5.8	3.8	5.3
gly	8.8	8.8	7.9
ala	8.5	8.9	7.8
cys	0.5	0.7	2.0
val	9.0	6.8	7.0
met	3.2	3.5	3.0
ile	5.2	6.8	6.1
leu	6.4	7.5	7.1
tyr	2.3	0.8	3.8
phe	4.9	5.1	5.2

NOTE: There are some discrepancies between the analyses of the subunits and that of the whole enzyme. These would probably be resolved if sufficient material was available to allow the compositions of the subunits to be determined from multiple analyses, as was the case with the whole enzyme.

TABLE 3.7

ESTIMATION OF THE MOLECULAR WEIGHTS OF THE SUBUNITS

PROTEIN	MOLECULAR WEIGHT	RELATIVE MOBILITY	REFERENCE
ferritin	18,500	0.97	Bryce & Crichton (1971)
trypsin	23,300	0.81	Hoffmann (1964)
aldolase	39,000	0.66	Lai *et al.* (1974)
catalase	60,000	0.46	Takeda *et al.* (1975)
phosphorylase b	94,000	0.29	Seery *et al.* (1967)
pyruvate carboxylase	120,000	0.21	Bais (1974)
propionyl-CoA carboxylase	58,000	0.49	
	72,000	0.40	

The proteins were electrophoresed in a 10% polyacrylamide-SDS slab gel using the discontinuous system of Laemmli *et al.* (1970).

FIGURE 3.1

PROPIONYL-CoA CARBOXYLASE AT VARIOUS STAGES OF PURIFICATION

Samples were electrophoresed in a 10% polyacrylamide-SDS gel using the discontinuous system of Laemmli (1970).

A. The pellet from 60% ammonium sulphate fractionation specific activity 0.12.

B. After DEAE ion exchange chromatography, specific activity 1.9.

C. After hydrophobic chromatography, specific activity 16.0.

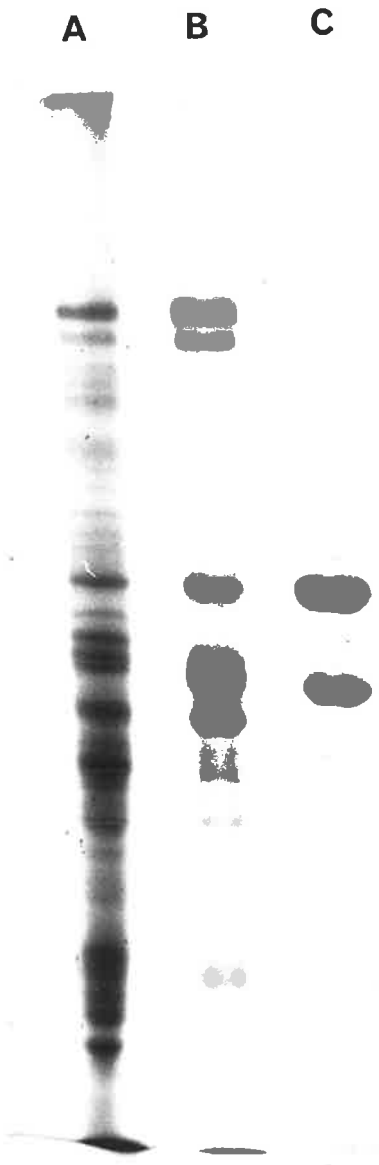
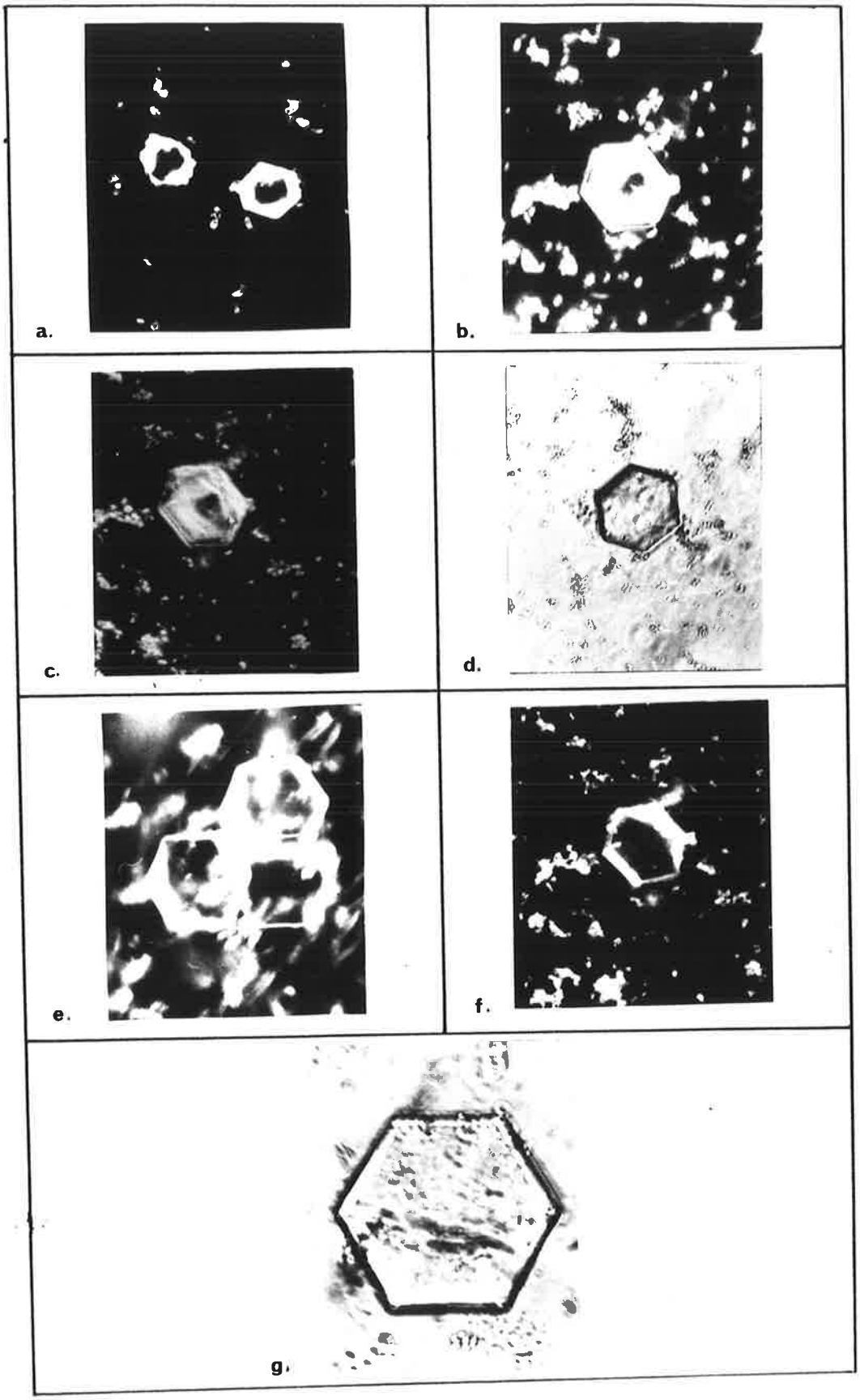

A
B
C

FIGURE 3.2

CRYSTALS OF PROPIONYL-CoA CARBOXYLASE

a - f magnification 167 X

g magnification 338 X.


a.
b.
c.
d.
e.
f.
g.

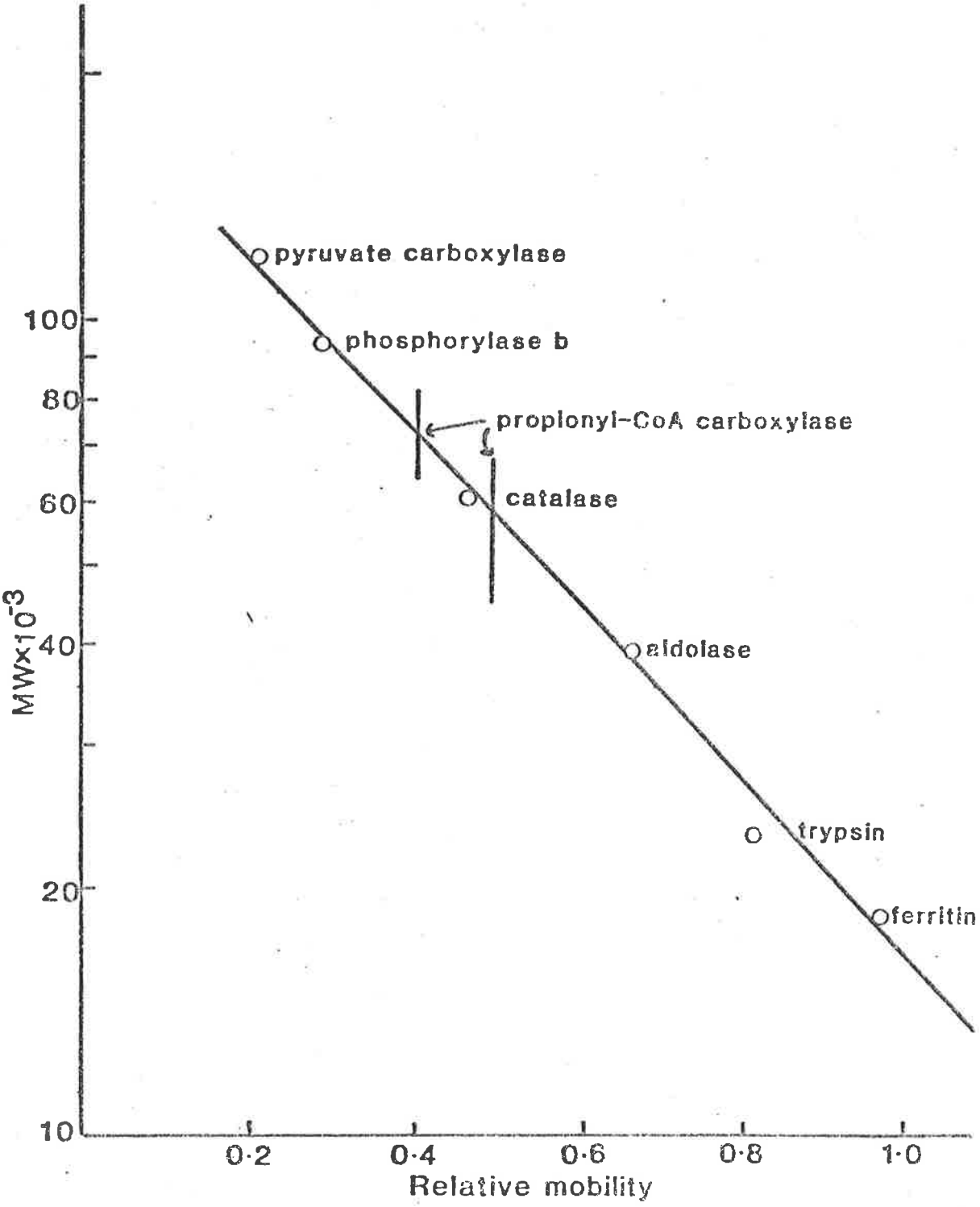


FIGURE 3.3

ESTIMATION OF THE MOLECULAR WEIGHTS OF THE SUBUNITS

The proteins indicated were electrophoresed in a 10% polyacrylamide-SDS slab gel using the discontinuous system of Laemmli *et al.* (1970). A single track containing all of the proteins was scanned in a spectrophotometer with a gel scanner attachment to accurately determine the relative mobilities.

FIGURE 3.4

ELECTROPHORESIS OF AVIDIN-TREATED PROPIONYL-CoA CARBOXYLASE

The samples were electrophoresed in a 10% polyacrylamide-SDS slab gel.

A. Propionyl-CoA carboxylase, 5 µg.

B. Propionyl-CoA carboxylase 5 µg, plus avidin 5 µg, mixed for 10 min at 25°C prior to electrophoresis.

The samples were not heated before being applied to the gel.

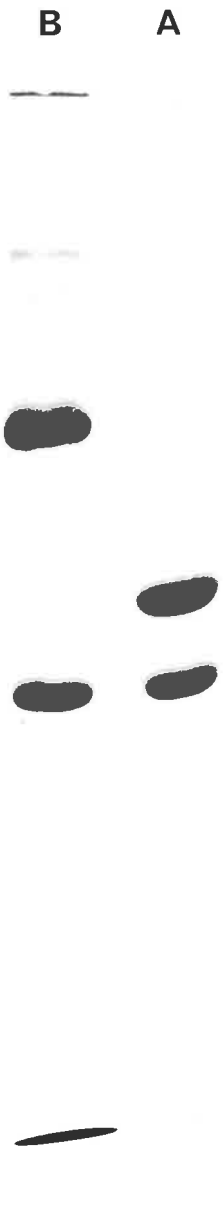

B
A

FIGURE 3.5

ELECTRON MICROGRAPH OF PROPIONYL-CoA CARBOXYLASE

The protein was prepared for electron microscopy as described in the Methods section.

Magnification 400,000 X.

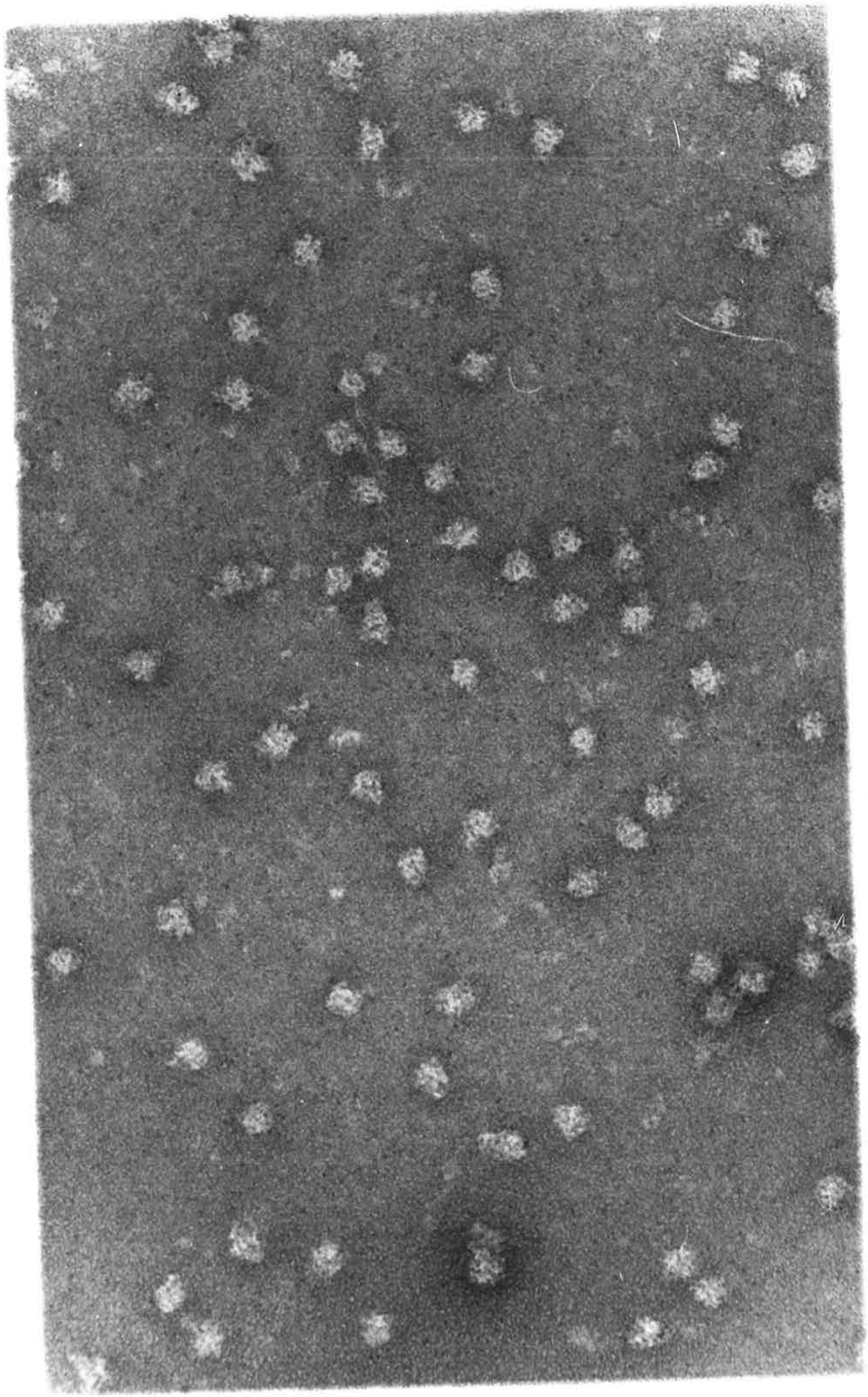
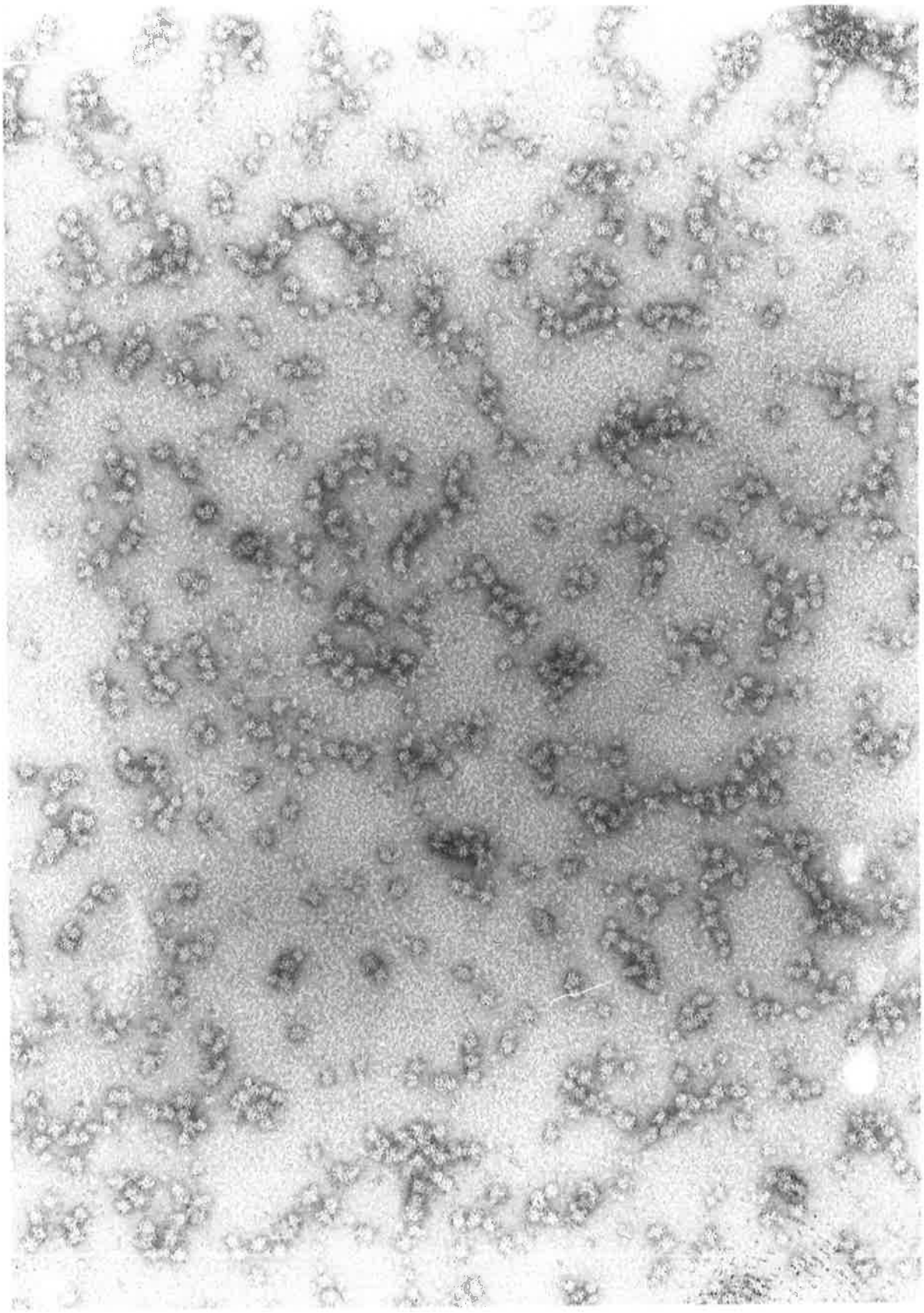


FIGURE 3.6

ELECTRON MICROGRAPH OF PROPIONYL-CoA CARBOXYLASE

The sample was prepared for electron microscopy as described in the Methods section.

Magnification 147,000 X.



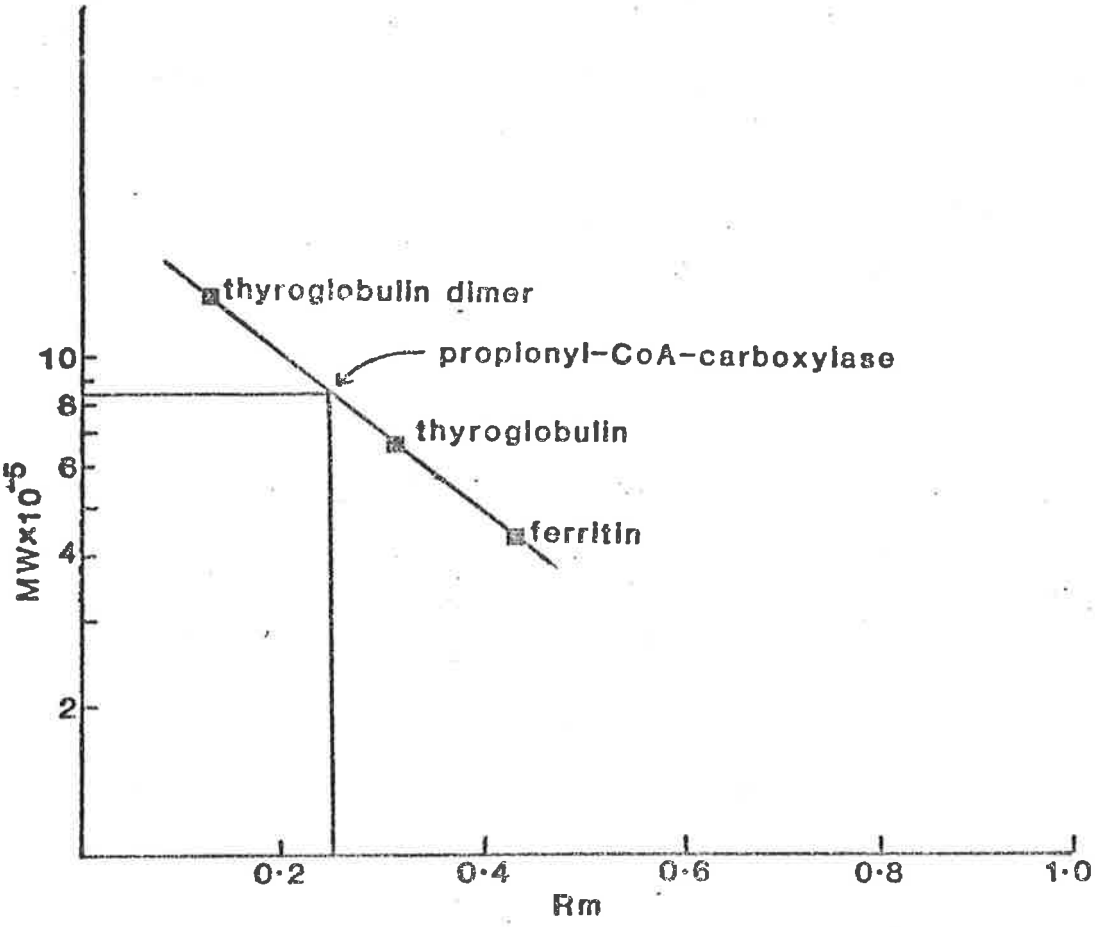


FIGURE 3.7

GEL ELECTROPHORESIS OF NATIVE ENZYME

Propionyl-CoA carboxylase and marker proteins (2.5 - 5 µg/protein) were electrophoresed on a 4 - 30% polyacrylamide gradient gel for 16 h at 150 V. The markers used were ferritin (440,000 daltons), thyroglobulin (669,000 daltons) and thyroglobulin dimer (1,338,000 daltons). The mobility of propionyl-CoA carboxylase indicates an apparent molecular weight of 840,000.

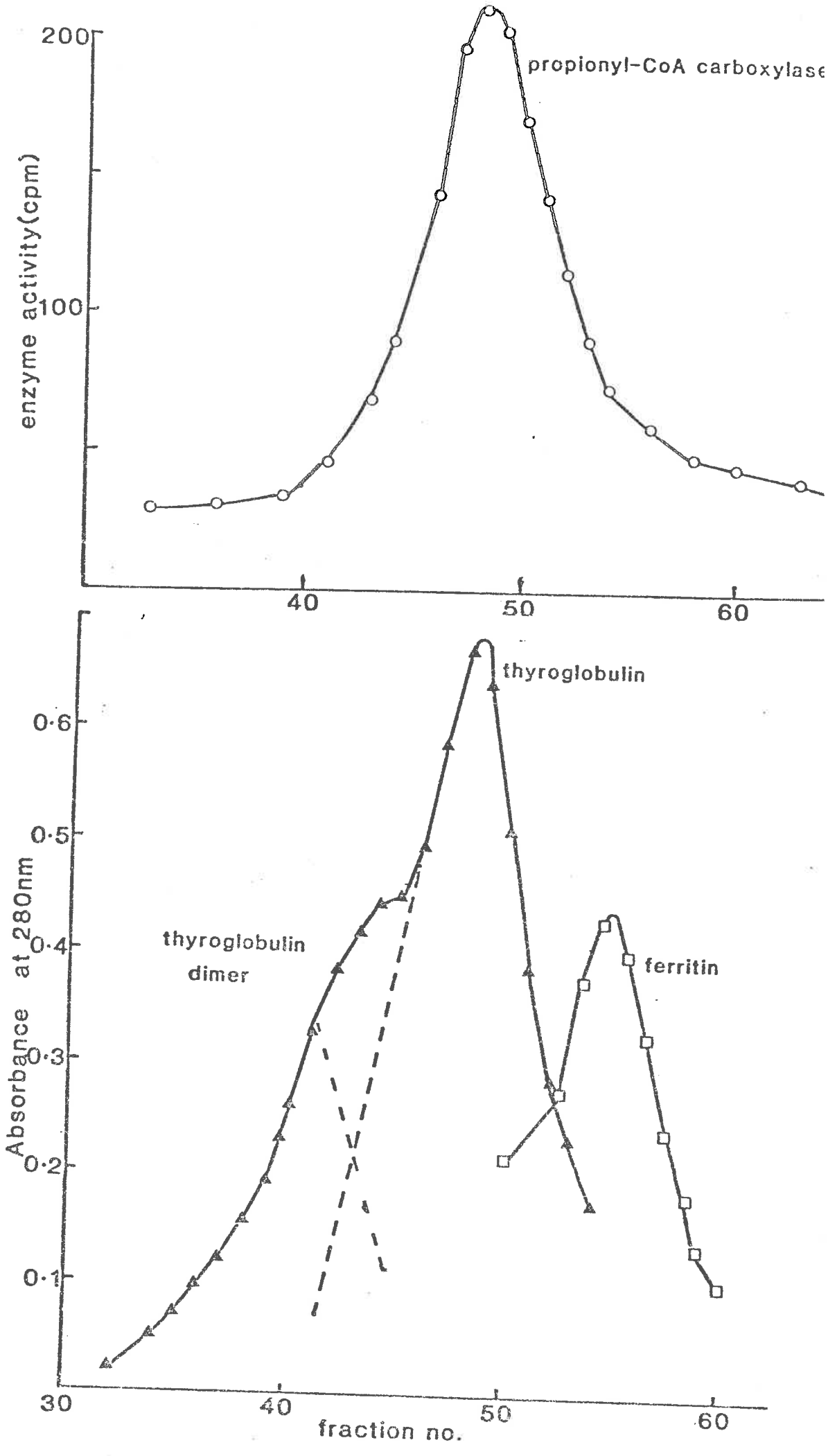

propionyl-CoA carboxylase
enzyme activity(cpm)
200
100
40
50
60
thyroglobulin
thyroglobulin dimer
ferritin
Absorbance at 280nm
0·6
0·5
0·4
0·3
0·2
0·1
30
40
50
60
fraction no.

FIGURE 3.8

GEL CHROMATOGRAPHY ON SEPHACRYL S-300

On separate occasions 1 ml of each of thyroglobulin (5 mg) ferritin (1 mg) or propionyl-CoA carboxylase (50 µg) was loaded on a 50 x 1.6 cm column of Sephacryl S-300. The column was eluted with 0.05 M N-ethylmorpholine-acetate buffer, pH 7.5, containing 1 mM EDTA and 0.5 mM DTE.

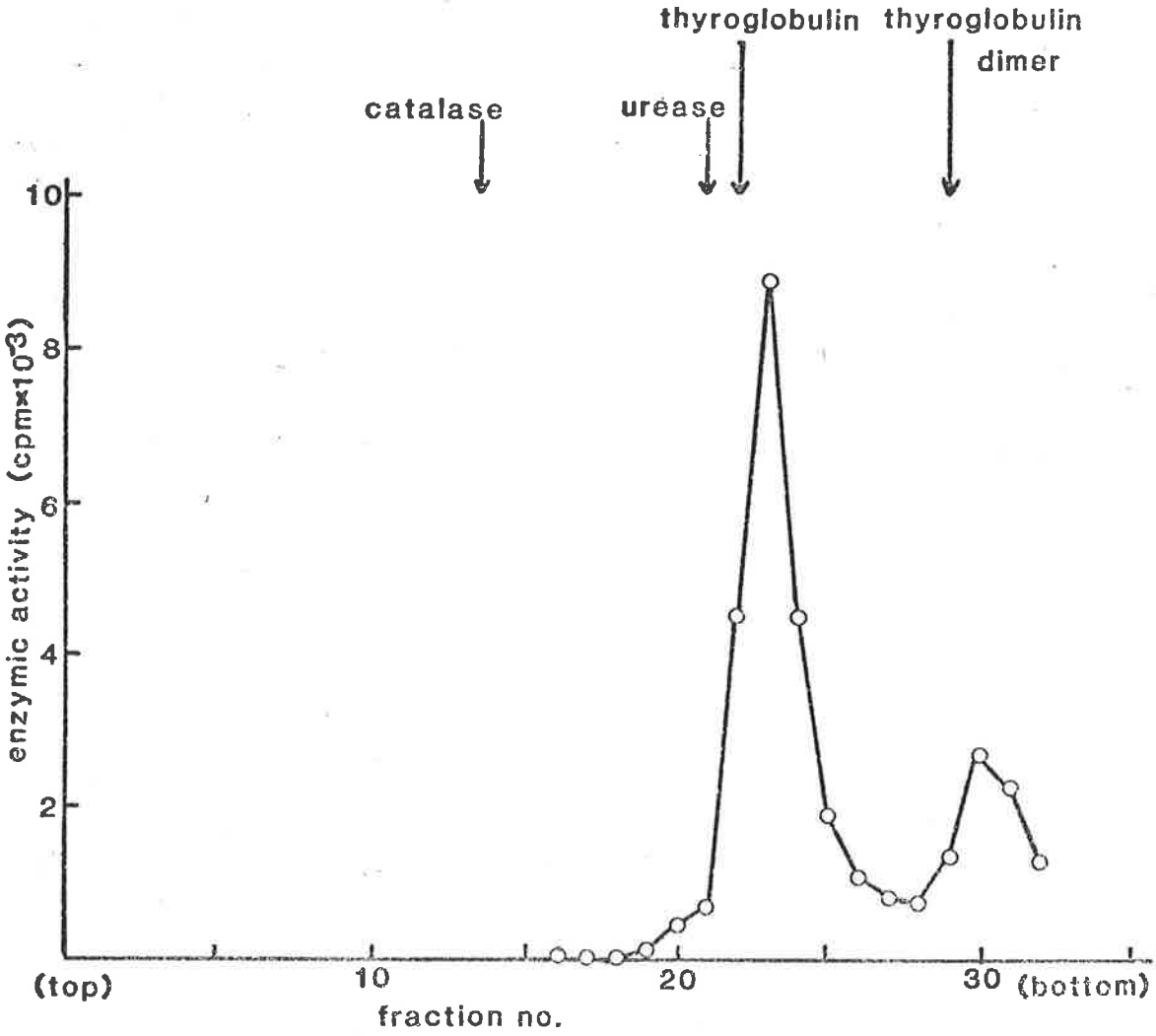


FIGURE 3.9

SUCROSE DENSITY GRADIENT SEDIMENTATION

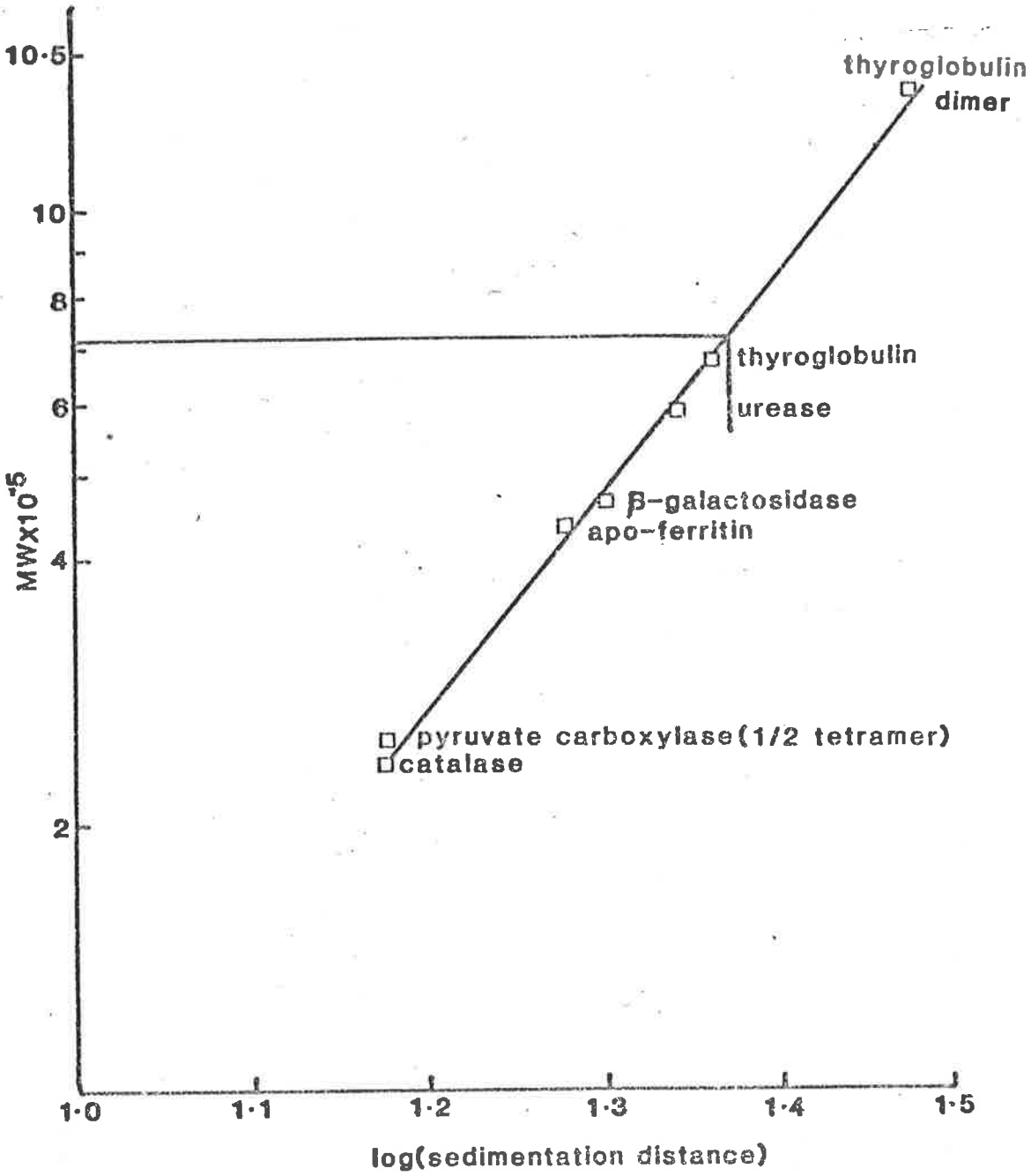
Samples were sedimented in 10 - 30% sucrose gradients in 50 mM KPi, 1 mM EDTA and 1 mM DTE, pH 7.2 at 4° for 15¼ h at 36,000 rpm in an SW-41 rotor. The samples were: propionyl-CoA carboxylase 0.17 mg; thyroglobulin (669,000 daltons) 1 mg; urease (590,000 daltons) 0.7 mg; catalase (232,000 daltons) 0.25 mg. The marker proteins were detected by absorbance at 280 nm.

FIGURE 3.10

SEDIMENTATION OF PURIFIED PROPIONYL-CoA CARBOXYLASE IN A SUCROSE DENSITY GRADIENT

Samples were sedimented in 10 - 30% linear sucrose gradients in 0.05 M N-ethylmorpholine-acetate, 1 mM EDTA, 1 mM DTE, pH 7.5 for 16½ h at 37,000 rpm and 4°C in an SW-41 rotor and then fractionated into 0.3 ml fractions with an Isco density gradient fractionater with 280 nm absorbance monitor. The following samples were sedimented: propionyl-CoA carboxylase (specific activity 11.0) 0.17 mg and 1.04 mg; thyroglobulin 1 mg; jack bean urease 1.05 mg; sheep liver pyruvate carboxylase 0.8 mg; β-galactosidase 0.1 mg; catalase 0.5 mg; apoferritin 1 mg. Pyruvate carboxylase, propionyl-CoA carboxylase and β-galactosidase were detected by assaying fractions for enzymic activity.

MARKER	MOLECULAR WEIGHT	REFERENCE
thyroglobulin	669,000	Edelhoch (1960)
urease	590,000	Dixon *et al.* (1980)
pyruvate carboxylase	480,000	Bais (1974)
β-galactosidase	465,000	Fowler & Zabin (1977)
apo-ferritin	440,000	Crichton *et al.* (1973)
catalase	232,000	Schroeder *et al.* (1969)


10·5
10
8
6
4
2
MWx10⁻⁵
thyroglobulin
dimer
thyroglobulin
urease
β-galactosidase
apo-ferritin
pyruvate carboxylase(1/2 tetramer)
catalase
1·0
1·1
1·2
1·3
1·4
1·5
log(sedimentation distance)

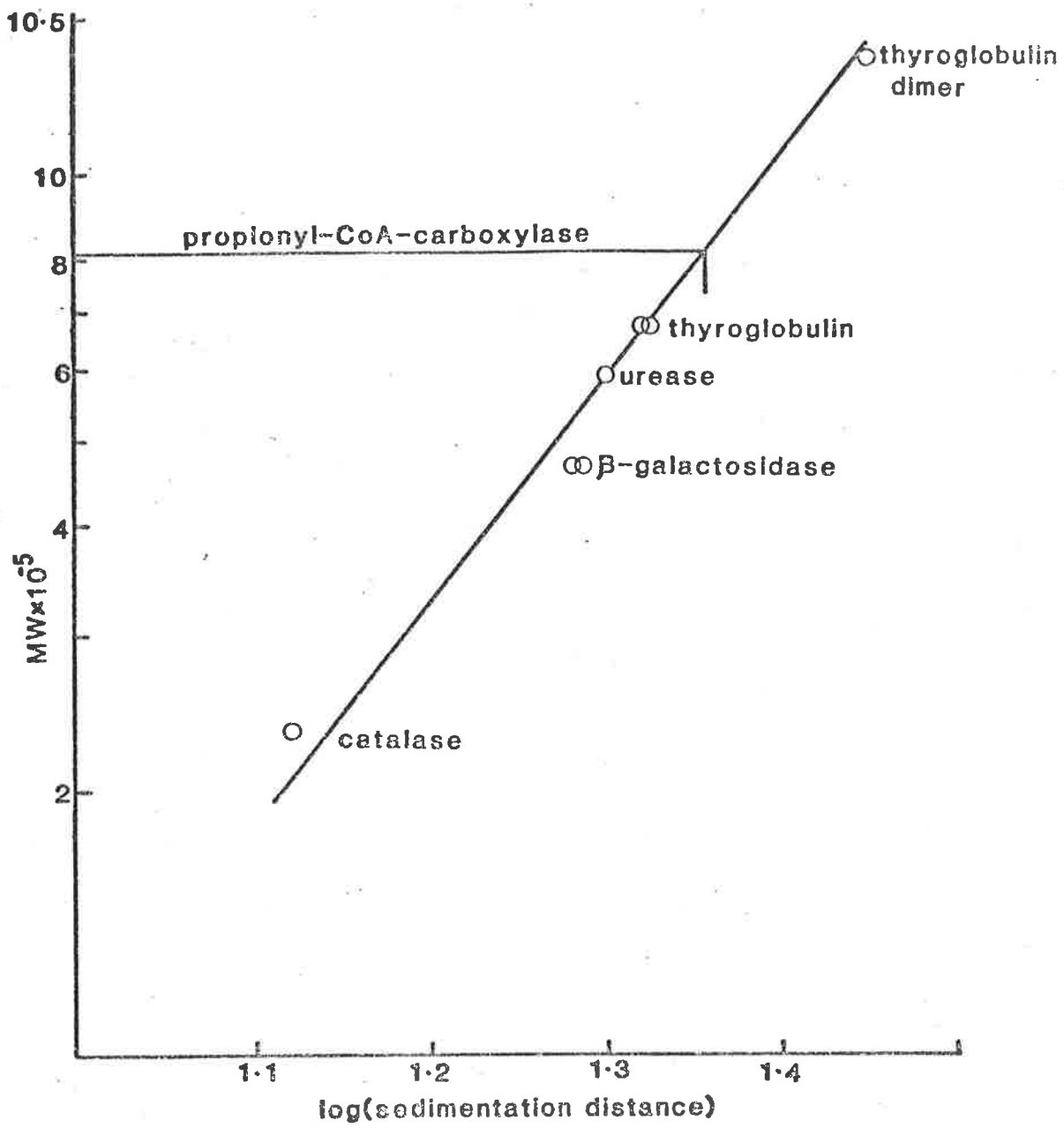


FIGURE 3.11

SUCROSE DENSITY GRADIENT SEDIMENTATION OF PROPIONYL-CoA CARBOXYLASE FROM A CRUDE MITOCHONDRIAL EXTRACT

A 0.5 ml sample from the initial extraction stage of the propionyl-CoA carboxylase purification procedure was sedimented in a 5 - 25% sucrose gradient in 0.1 M N-ethyl-morpholine-HCl, 1 mM EDTA, 1 mM DTE, pH 7.8, at 5°C for 10 h at 37,000 rpm in an SW-41 rotor. The marker proteins and fractionation were the same as for Fig. 3.10, except that apo-ferritin and pyruvate carboxylase were not used.

CHAPTER 4

GROUP SPECIFIC MODIFICATION AND AFFINITY LABELLING OF PROPIONYL-CoA CARBOXYLASE

4.1 INTRODUCTION

The biotin-dependent carboxylases all have at least three functional components. In the tripartite acetyl CoA carboxylase of *E. coli* the three functions reside on separate monofunctional polypeptides, called biotin carboxylase, biotin carboxyl carrier protein (BCCP), and carboxyltransferase. In the monopartite acetyl-CoA carboxylase found in mammals, the three functions are all carried out by a single, multifunctional polypeptide. Perhaps during the evolution of the mammalian enzyme three genes have been fused into a single gene.

In Section 1.1.4 I have reviewed the evidence for and against the proposal that some functional components of different biotin-dependent enzymes have a common origin. It is possible that a primordial biotin carboxylase gene that was fused with a BCCP gene was, on several occasions, duplicated and spliced with a gene coding for a keto acid- or acyl-CoA-binding protein, each time producing a monopartite biotin-dependent carboxylase with a different acceptor substrate specificity. [Gilbert (1975) has suggested that one of the functions of introns in eukaryotic genes is to facilitate the evolution of new proteins by the splicing of genes or parts of genes.] Alternatively, the monofunctional biotin carboxylase and biotin carboxyl carrier proteins may have evolved to associate with a range of keto acid- and acyl-CoA-binding proteins, producing tripartite biotin-dependent carboxylases.

Lynen (1975) suggested that the variety of structural features seen in biotin-dependent enzymes represent various

stages in the development, by gene fusion, of multifunctional polypeptides, which are superior from the kinetic as well as the regulatory point of view. If Lynen is correct, then bipartite propionyl-CoA carboxylase would represent an intermediate stage in this evolutionary pathway and we would expect the two catalytic activities of the enzyme to reside on different polypeptides, one of which has fused with the BCCP.

To test whether Lynen's hypothesis applies to propionyl-CoA carboxylase, a group-specific reagent and two potential affinity labels have been used in this investigation, as probes for the active sites. Edwards & Keech (1967) have reported the modification by N-ethylmaleimide of a single sulphydryl group involved in the binding of propionyl-CoA to pig heart propionyl-CoA carboxylase. They found the reaction between N-ethylmaleimide and the enzyme to be pseudo-first order with respect to inhibitor concentration and time.

The 2',3'-dialdehyde derivative of ATP (oATP) has been successfully employed as an affinity label of the MgATP binding site of sheep liver pyruvate carboxylase (Easterbrook-Smith *et al.*, 1976). The success of this method relies on the availability of a lysine residue at the ATP binding site. A Schiff's base forms between the lysine ε-amino group and an aldehyde of the oATP. Subsequent reduction with sodium borohydride converts the unstable Schiff's base to a stable C-N bond, thereby covalently attaching the nucleotide derivative to the enzyme. A 2',3'-dialdehyde derivative can also be made from dephospho-

propionyl-CoA and is a potential affinity label for the propionyl-CoA site.

4.2 METHODS

4.2.1 Treatment of the enzyme with N-ethylmaleimide

Propionyl-CoA carboxylase (2.7 units, specific activity 16) was mixed with 1 mM N-ethylmaleimide in 100 mM sodium-morpholinopropanesulphonate buffer, pH 7.0, 10 mM $MgCl_2$ and, where present, 2 mM propionyl-CoA, in a volume of 0.5 ml at 25°C. At various time intervals, aliquots of 10 µl were removed and assayed for enzymic activity.

4.2.2 Subunit labelling with N-ethylmaleimide

The inactivating solution contained 1.1 units of enzyme (S.A.11), 2.5 mM $MgCl_2$, 100 mM sodium morpholino-propanesulphonate, pH 7.0, 0.1 mM N-[^{14}C]ethylmaleimide (10 mCi/mmole) and where present, 2.8 mM propionyl-CoA, in a volume of 0.12 ml at 22°C. Aliquots of 30 µl were removed at 5, 10 and 20 min and added to 1 ml of ice-cold 15% tri-chloroacetic acid containing 1 mM glutathione to quench any remaining N-ethylmaleimide. The protein was collected by centrifuging and washed three times with 1 ml of 3:1 ether-acetone solution. Finally, the samples were dissolved in 20 µl of electrophoresis loading solution and subjected to electrophoresis in 6 mm diameter SDS-polyacrylamide tube gels. After staining and destaining the gels were cut into 1 mm sections and soaked for 16 h at 25°C in 0.2 ml of 90% NCS solubilizer. After adding 3 ml of scintillation fluid the radioactivity in each section was measured.

4.2.3 Synthesis of oATP

oATP was prepared and purified by the method of Easterbrook-Smith *et al.* (1976b). ATP (0.1 mmol) was dissolved in 0.9 ml of water and the solution adjusted to pH 7.0 with NaOH. Sodium periodate (0.11 mmol) was added and the solution allowed to stand at 4°C in the dark for 1 h. After this time the reaction was complete as judged by chromatography on polyethyleneimine thin layers developed with 0.8 M NH_4HCO_3. The reaction was stopped by the addition of 5 µl of ethylenediol and the reaction mixture then loaded onto a Sephadex G-10 column (25 x 2.4 cm) previously equilibrated with distilled water at 4°C. The column was eluted with distilled water and the leading half of the nucleotide peak was pooled, freeze-dried and stored at -80°C. The concentration of oATP was determined by measuring the absorbance at 258 nm, using a value of 14,900 cm^{-1} M^{-1} for the absorption co-efficient (Hansske *et al.*, 1974). The purity of the oATP was confirmed by chromatography in two different solvent systems. Thin layer chromatography was performed on polyethyleneimine sheets, using 0.8 M NH_4HCO_3 as the developing solvent. Ultraviolet light was used to locate the position of the nucleotide (Rf. 0.02). Ascending paper chromatography was performed on Whatman 3 MM paper, using 1-butanol/acetic acid/water (4:1:5, by vol) as the developing solvent (Rf. 0.10). In each case only one compound was detected.

4.2.4 Synthesis of o-dephosphopropionyl-CoA

[^{3}H]CoA (260 nmol, 76 mCi/mmole) was converted to propionyl-CoA and then freeze-dried and redissolved in 250 µl of 0.1 M Tris-HCl, pH 7.4 containing 2 units of 3' nucleotidase. After 6 h at 25°C the sample was diluted with 3 volumes of 3 mM HCl and loaded on a column of DEAE-cellulose (10 x 1.4 cm) which had been equilibrated with 3 mM HCl. The dephosphopropionyl-CoA was eluted with a 200 ml gradient of 0.08 - 0.30 M LiCl in 3 mM HCl. A minor nucleotide peak co-chromatographed with propionyl-CoA on polyethyleneimine thin layers with 0.4 M NH_4HCO_3 as developing solvent (Rf. 0.50). The major nucleotide peak was more mobile in the same chromatography system (Rf. 0.69), as expected of dephosphopropionyl-CoA. Fractions containing the major peak were freeze-dried and then desalted on a column of Sephadex G-10 (60 x 1.6 cm). The dephosphopropionyl-CoA was oxidized with periodate by a method similar to that used for the synthesis of oATP.

4.2.5 Covalent modification of propionyl-CoA carboxylase with oATP and o-dephosphopropionyl-CoA

The modifications were carried out at 25°C in 28 µl of a solution which contained propionyl-CoA carboxylase (43 µg, specific activity 7.8), 54 mM N-ethylmorpholine-acetate, pH 7.2, 1 mM $MgCl_2$ and either 0.5 mM o[^{14}C]ATP (61,000 cpm per nmol) or 0.5 mM o[^{3}H]dephosphopropionyl-CoA (76,000 cpm per nmole). Where indicated, the solution also contained either 15 mM MgATP or 15 mM Mg-propionyl-CoA. After 2 min incubations, 2.3 µl of 100 mM sodium

borohydride was added and again after 5 min. After a further 20 min, 14 µl of 3X-concentrated SDS-electrophoresis loading solution was added, the samples boiled for 2 min and then electrophoresed in 10% polyacrylamide-SDS tube gels (0.5 x 8 cm). After staining and destaining, the gels were scanned with a Varian 635-D spectrophotometer with a gel scanner attachment and then cut into 1 mm sections in the region of the propionyl-CoA carboxylase subunits. Each slice was incubated in 0.3 ml of 90% NCS solubilizer at 37°C for at least 4 h. When all of the slices were colourless, 3 ml of scintillation fluid was added and the radioactivity measured.

4.3 RESULTS

4.3.1 Inhibition by N-ethylmaleimide

To check that propionyl-CoA carboxylase from sheep liver is inactivated by the sulphydryl reagent N-ethyl-maleimide in a similar way to the enzyme from pig heart (Edwards & Keech, 1967), the rate of inactivation was measured in the presence and absence of propionyl-CoA. In the absence of propionyl-CoA half of the activity was lost within 12 min, while in the presence of propionyl-CoA 20% of the activity was lost in 12 min (Fig. 4.1). The N-ethyl-maleimide interacted with sheep liver propionyl-CoA carb-oxylase in a way similar to that observed by Edwards & Keech with the pig heart enzyme, although the enzyme appeared to retain some residual activity. Since Edwards & Keech restricted the inactivation times to less than 15 min, they

would not have observed the residual activity, or a large deviation from linearity in plots of log (% of initial activity vs time. It has been pointed out by Dr. R.L. Blakeley (personal communication) that the residual activity may have been due to masking of the reactive sulphydryl group in the form of a mixed disulphide, which would be reduced in the assay system by DTE. Edwards & Keech circumvented such a possibility by including 1 mM glutathione in their inactivation system. In constructing the log (% (activity at t=0 - activity at t=∞)) vs time plot, shown in Fig. 4.1.b, the activity at t=∞ was assumed to be 35% of the initial activity.

In a similar experiment, but over a shorter time period, ATP was found to have little effect on the rate of inactivation unless propionyl-CoA was also present. There was greater protection by ATP plus propionyl-CoA than by propionyl-CoA alone (Fig. 4.2). Although the rate of inactivation was observed to slow down when monitored over a long time period, an estimate of the residual activity at t=∞ was not obtained and so the data is plotted in the form of log (% of initial activity) vs time.

4.3.2 Subunit labelling with N-ethylmaleimide

In order to determine which of the subunits was being modified, propionyl-CoA carboxylase was reacted with N-[^{14}C]ethylmaleimide and the subunits separated by SDS-polyacrylamide gel electrophoresis. The labelling patterns are shown as histograms in Fig. 4.3 and as time courses in Fig. 4.5. In the absence of propionyl-CoA the larger subunit contains 2.3 - 2.6 times the radioactivity found in the smaller subunit, and incorporation was still occurring at 20 min. In the presence of propionyl-CoA much less radioactivity is incorporated into both subunits, with no increase after 5 min. The larger subunit contains 3 times the

radioactivity found in the smaller subunit. Propionyl-CoA protects both subunits from modification, apart from a small amount of rapid incorporation. (Propionyl-CoA was added to the enzyme before N-ethylmaleimide.) Similar results were obtained when the modification was performed at pH 8.0 in Tris-HCl buffer.

In case the modification observed in the presence of propionyl-CoA was occurring mainly at sites other than the binding site for propionyl-CoA, the experiment was repeated, but this time the enzyme was first reacted with non-radioactive N-ethylmaleimide in the presence of propionyl-CoA. Three units of enzyme in 0.1 M morpholinopropanesulphonic acid, pH 7.0, was mixed with 3 mM propionyl-CoA, 3 mM $MgCl_2$ and 0.1 mM N-ethylmaleimide at 22°C. After 5 min the small molecules were removed by centrifugal desalting. The chemically modified enzyme was then subjected to modification with N-[^{14}C]ethylmaleimide in the presence and absence of propionyl-CoA under the same conditions as used for the modification of untreated enzyme. In this case, radioactivity was incorporated into the smaller subunit at a faster rate, both in the presence and absence of propionyl-CoA (Figs. 4.4 and 4.5). Again there was less radioactivity incorporated into both subunits when propionyl-CoA was present. It seems that during the initial modification with non-radioactive N-ethylmaleimide the reactive sulphydryls of the larger subunit were preferentially modified so that there were fewer readily available for subsequent modification with the radioactive label.

These experiments implicate both subunits in the binding of propionyl-CoA. The reaction of N-ethylmaleimide with sheep liver propionyl-CoA carboxylase does not seem to be as specific as with the pig heart enzyme. In order to further investigate the substrate binding sites on the enzyme use was made of affinity labels, which could be more specific

for the active sites than a group-specific label such as N-ethylmaleimide.

4.3.3 Inhibition by oATP

Fig. 4.6 shows the inactivation of propionyl-CoA carboxylase by the 2',3'-dialdehyde derivative of ATP (oATP). The enzyme-inhibitor complex was relatively stable so that negligible reactivation occurred during the subsequent assay procedure, even when the complex was not reduced by sodium borohydride. In the absence of substrates, less than 5% of the initial activity remained after 15 min. The addition of 10 mM bicarbonate to the basic inactivation system had no effect on the rate of inactivation while 1 mM ATP and 1 mM propionyl-CoA each decreased the rate of inactivation to a similar extent. Only the points obtained in the presence of 10 mM bicarbonate are shown.

When a lower concentration of oATP was used (0.22 mM) only partial inactivation occurred (Fig. 4.7). In the absence of substrates, at least 75% of activity was lost, but in the presence of 1 mM MgATP the activity dropped to 60% in 10 min, but remained constant thereafter. Propionyl-CoA only provided slight protection and this protection was additive with the protection by MgATP.

The partial protection provided by propionyl-CoA can be interpreted in three different ways. Firstly, it is possible that oATP binds to both the MgATP and the propionyl-CoA binding sites, but preferentially to the MgATP site. Secondly, the binding of propionyl-CoA to its binding site may induce a change in the environment of the MgATP binding site such that the affinity of oATP for this site is reduced. Alternatively, propionyl-CoA may bind at the MgATP site to

some extent and so compete with oATP. The third possibility seems unlikely as no substrate inhibition by propionyl-CoA was observed at the concentrations used in these experiments.

4.3.4 Subunit labelling by oATP

Propionyl-CoA carboxylase was modified with o[^{14}C]-ATP in the presence of sodium borohydride and the subunits separated by SDS-polyacrylamide gel electrophoresis. The larger subunit contained 72% of the radioactivity incorporated (Fig. 4.8a). When the modification was carried out in the presence of either MgATP (Fig. 4.8b) or propionyl-CoA (Fig. 4.8c) there was less incorporation into both subunits, however, the proportion of radioactivity associated with each subunit was not altered significantly by the presence of the substrates. The larger subunit contained 58% in the presence of propionyl-CoA and 66% in the presence of MgATP. On the basis of this evidence it is not possible to make an unequivocal assignment of the ATP binding site to either subunit.

4.3.5 Subunit labelling with o-dephosphopropionyl-CoA

The periodate oxidation product of dephosphopropionyl-CoA (o-dephosphopropionyl-CoA) was used to covalently modify propionyl-CoA carboxylase in the same way that oATP was used. The o-dephosphopropionyl-[^{3}H]CoA modified both subunits, but in the absence of substrates more of the label (71%) was attached to the larger subunit (Fig. 4.9a). When the modification reaction was carried out in the presence

of MgATP or propionyl-CoA (Fig. 4.9b,c) there was less incorporation into the larger subunit while the amount of label attached to the smaller subunit was not much affected.

4.4 DISCUSSION

The results of these modification studies on propionyl-CoA carboxylase indicate that propionyl-CoA affects the MgATP binding site and conversely, MgATP affects the propionyl-CoA binding site. On the one hand, MgATP does not protect against inhibition by N-ethylmaleimide, which reacts at the propionyl-CoA binding site (Edwards & Keech, 1967), but MgATP does increase the protection given by propionyl-CoA. On the other hand, propionyl-CoA provides some protection against inactivation by the ATP analogue oATP. There are several ways these effects could occur. ADP is a component structure of both ATP and propionyl-CoA and so it is quite possible that each can bind at the other's binding site. They could even share the same binding site. If this remote possibility were the case the enzyme should have a classical ping-pong kinetic mechanism and so it can be easily tested. Alternatively, the binding of each substrate could induce changes in the environment of the other substrate's binding site. Further evidence for this point comes from the work of Hegre & Lane (1966) who found that MgATP facilitates the reaction of p-chloromercuribenzoate at the propionyl-CoA site of bovine liver propionyl-CoA carboxylase. A fourth possibility is that the inhibitors may

react at both the ATP and the propionyl-CoA binding sites.

The effect of oATP on propionyl-CoA carboxylase is similar to its effect on pyruvate carboxylase, in that MgATP and acetyl-CoA both protect pyruvate carboxylase against modification (Easterbrook-Smith *et al.*, 1976). However, oxidized dephosphoacetyl-CoA could not be reduced onto pyruvate carboxylase.

Similar subunit labelling patterns were observed when propionyl-CoA carboxylase was modified with N-ethylmaleimide, oATP and o-propionyl-CoA. In each case the larger subunit was preferentially labelled but the smaller subunit was also labelled to some extent. Propionyl-CoA and MgATP each protected both subunits against modification, although the larger subunit was better protected in each case (except that protection by MgATP against subunit modification by N-ethylmaleimide was not tested). One possible explanation for these results is that MgATP and propionyl-CoA both bind in a crevice between the two subunits, with both subunits contributing to the binding sites. Each subunit must then supply a lysine residue in both the MgATP and the propionyl-CoA sites which can be modified by oATP and o-dephospho-propionyl-CoA respectively. Each subunit must also supply a cysteine sidechain for the N-ethylmaleimide to react with, although the slow labelling of the smaller subunit by N-ethylmaleimide could conceivably be due to modification of a lysine sidechain with an unusually low pKa.

A straightforward interpretation of these enzyme modification experiments cannot be made. However, none of the results obtained support the hypothesis that the ATP and

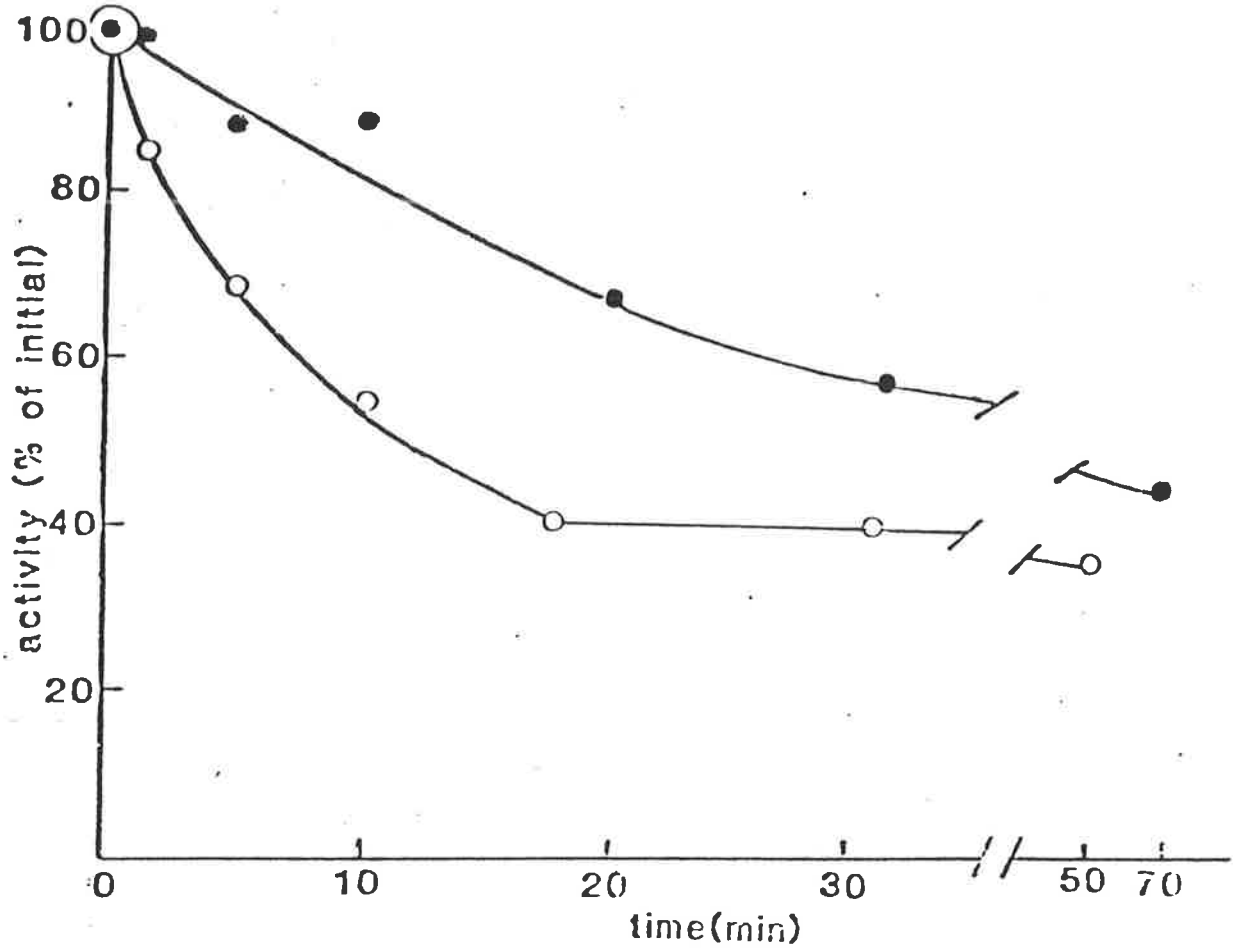
propionyl-CoA binding sites are on different subunits. In the light of the finding that both the large and small subunits react similarly with three different modifiers, it is interesting to note that the subunits have very similar amino acid compositions (See Chapter 5).

FIGURE 4.1

INACTIVATION BY N-ETHYLMALEIMIDE

A. The inactivation mixture contained 2.7 units of enzyme (S.A.16), 1 mM N-ethylmaleimide, 10 mM $MgCl_2$, 0.1 M sodium morpholinopropanesulphonic acid, pH 7.0, and no (O) or 2 mM (●) propionyl-CoA, in a volume of 0.5 ml at room temperature. Aliquots of 10 µl were assayed for enzymic activity at the designated time intervals.

B. The data of Fig. 4.1A replotted on a semi-log scale where Act_t and Act_∞ represent enzymic activity at time t and infinity.


100
80
60
40
20
activity (% of initial)
0
10
20
30
50
70
time(min)

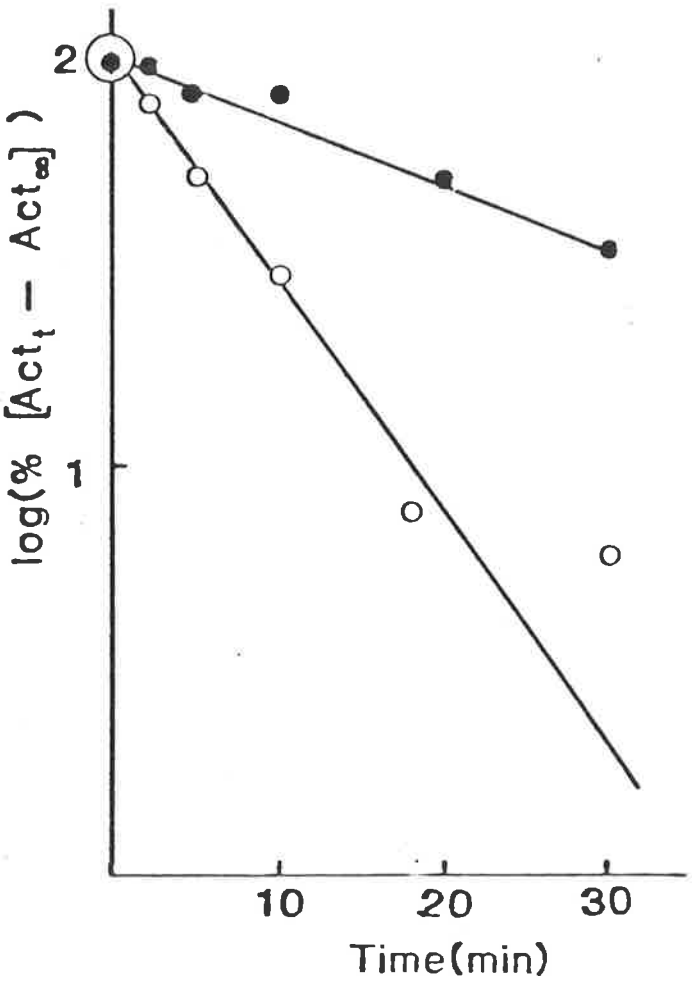
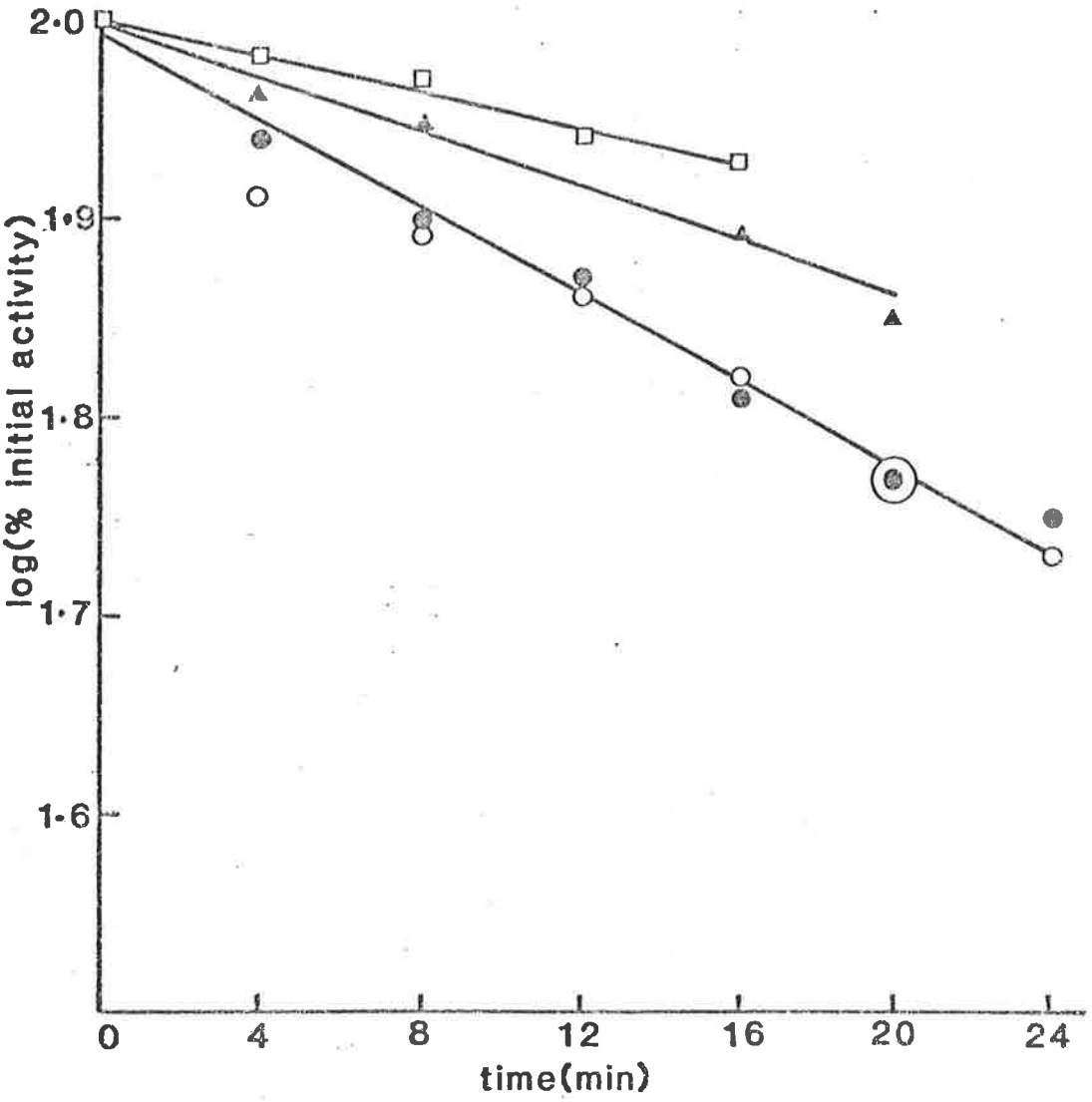

2
1
log(% [Act$_t$ − Act$_\infty$])
10
20
30
Time(min)

FIGURE 4.2

PROTECTION AGAINST N-ETHYLMALEIMIDE INACTIVATION BY PROPIONYL-CoA AND ATP

Enzyme (S.A.14) was treated with 4 mM DTE for 30 min before being transferred to 0.1 M morpholinopropanesulphonic acid buffer, pH 7.0, by centrifugal desalting. After preincubation at 28°C in the presence of 2 mM $MgCl_2$ (●); 5 mM ATP and 7 mM $MgCl_2$ (○); 2 mM propionyl-CoA and 6 mM $MgCl_2$ (▲); or 2 mM propionyl-CoA, 5 mM ATP and 8 mM $MgCl_2$ (□). N-ethylmaleimide was added to a final concentration of 1 mM and 10 µl samples assayed for activity at the designated times. The $MgCl_2$ concentrations were chosen to maintain a constant concentration of free Mg^{2+}.

The inactivation rate^constant was determined from the slope of a plot of ln (% of initial activity) against time using the GLIM program with log "link" (Baker & Nelder, 1978).



ADDITIONS	$k_{inact.}(min^{-1})$
none	0.025 ± 0.001
MgATP (5 mM)	0.024 ± 0.003
propionyl-CoA (2 mM)	0.016 ± 0.002
MgATP (5 mM) + propionyl-CoA (2 mM)	0.011 ± 0.001

FIGURE 4.3

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF N-[^{14}C]ETHYLMALEIMIDE-LABELLED PROPIONYL-CoA CARBOXYLASE

The enzyme (1.1 units, S.A.11) was modified with 0.1 mM N-[^{14}C]ethylmaleimide (10 mCi/mmole) in 0.1 M sodium morpholinopropanesulphonate, pH 7.0, 2.5 mM $MgCl_2$ and, where present, 2.8 mM propionyl-CoA in a volume of 0.12 ml at 22°C. Aliquots of 30 µl were removed at the times indicated and added to 1 ml of 15% trichloroacetic acid containing 1 mM glutathione at 0°C. After the precipitated protein was washed with 3:1 ether:acetone, it was subjected to SDS-gel electrophoresis in 8% polyacrylamide tube gels (6 mm). The stained gels were cut into 1 mm sections and the radioactivity associated with the subunits was measured.

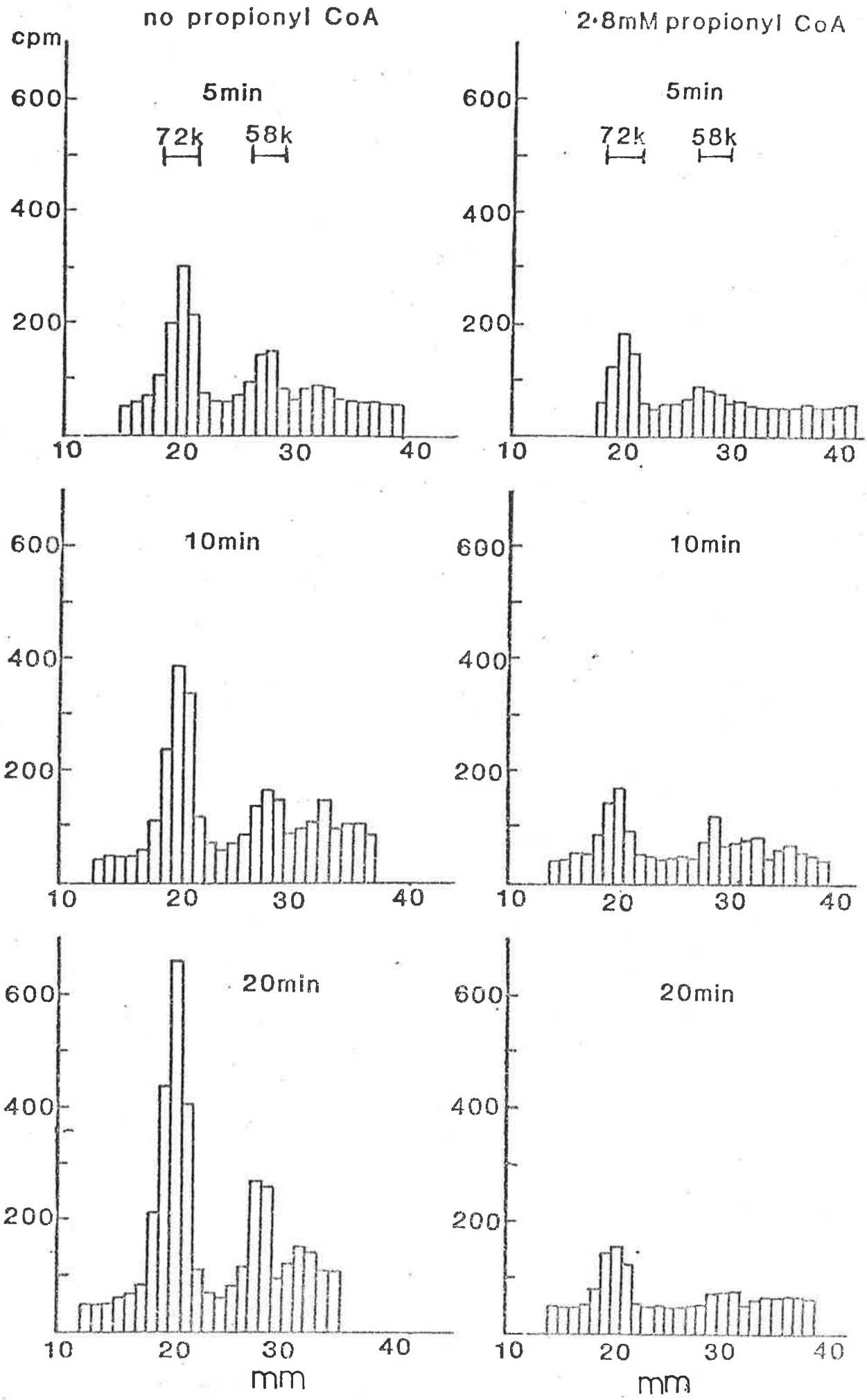

no propionyl CoA
2·8mM propionyl CoA
cpm
5min
72k
58k
10min
20min
600
400
200
10
20
30
40
mm

FIGURE 4.4

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF ENZYME WHICH WAS BRIEFLY MODIFIED WITH NON-RADIOACTIVE N-ETHYLMORPHOLINE IN THE PRESENCE OF PROPIONYL-CoA, BEFORE LABELLING WITH N-[^{14}C]ETHYLMALEIMIDE

Enzyme was subjected to modification with 0.1 mM N-ethylmaleimide in the presence of 3 mM propionyl-CoA, 3 mM $MgCl_2$ and 0.1 M sodium morpholinopropanesulphonate, pH 7.0, for 5 min at 22°C. After isolation of the enzyme by centrifugal desalting it was subjected to labelling with N-[^{14}C]ethylmaleimide and gel electrophoresis, using the conditions described in the legend to Fig. 4.3.

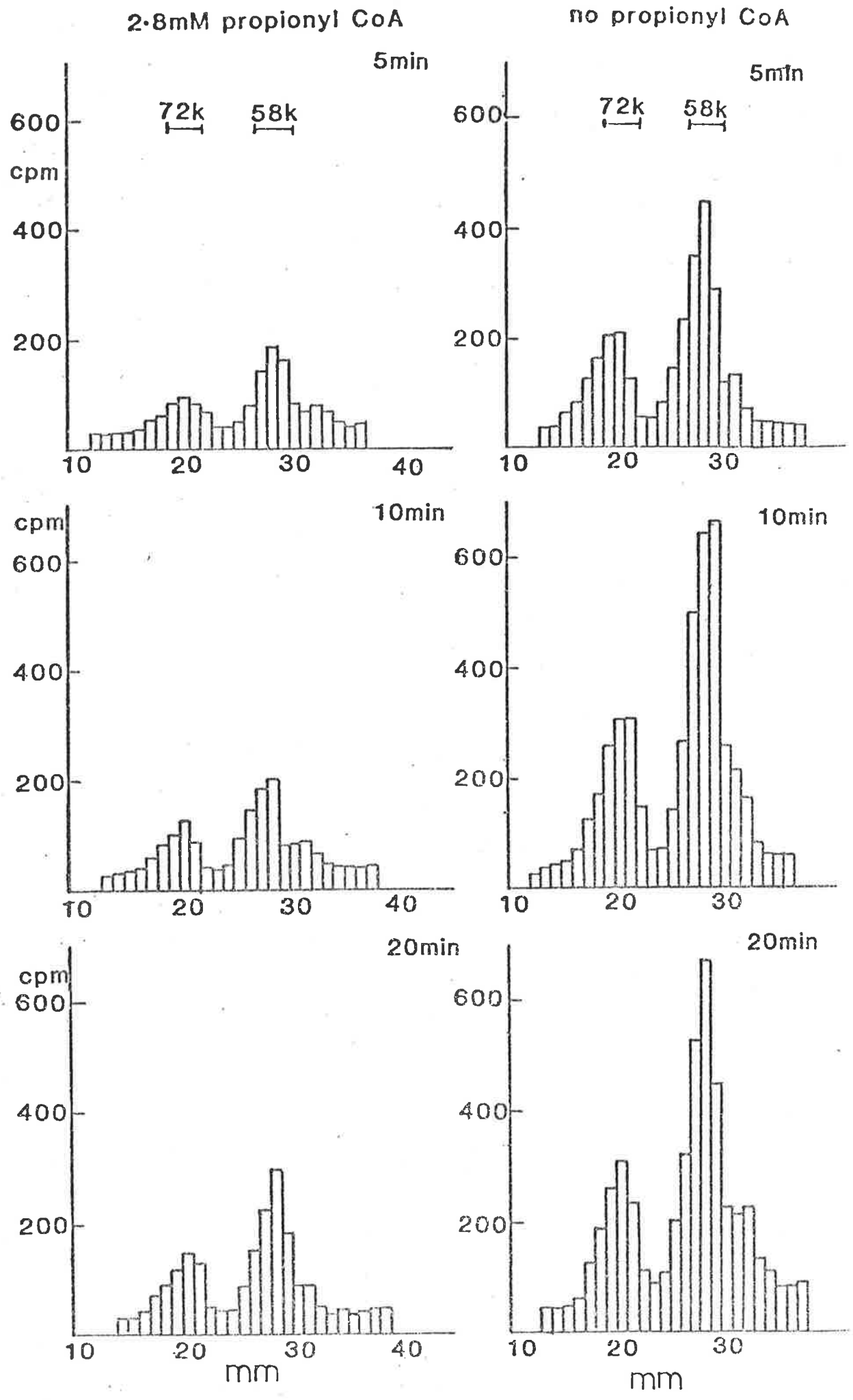

2·8mM propionyl CoA
no propionyl CoA
5min
5min
72k
58k
72k
58k
600
400
200
cpm
10
20
30
40
10min
10min
20min
20min
mm
mm

FIGURE 4.5

TIME-COURSE OF THE LABELLING OF THE SUBUNITS WITH N-[^{14}C]ETHYLMALEIMIDE

The amount of radioactivity incorporated into each subunit has been calculated from the areas under the relevant peaks in Figs. 4.3 and 4.4 Large subunit (▲), small subunit (△).

(a) Labelling in the absence of propionyl-CoA

(b) Labelling in the presence of propionyl-CoA

(c) Prior modification with non-radioactive N-ethylmaleimide in the presence of propionyl-CoA, followed by labelling with N-[^{14}C]ethyl-maleimide in the absence of propionyl-CoA

(d) Prior modification with non-radioactive N-ethylmaleimide in the presence of propionyl-CoA, followed by labelling and N-[^{14}C]ethyl-maleimide in the presence of propionyl-CoA.

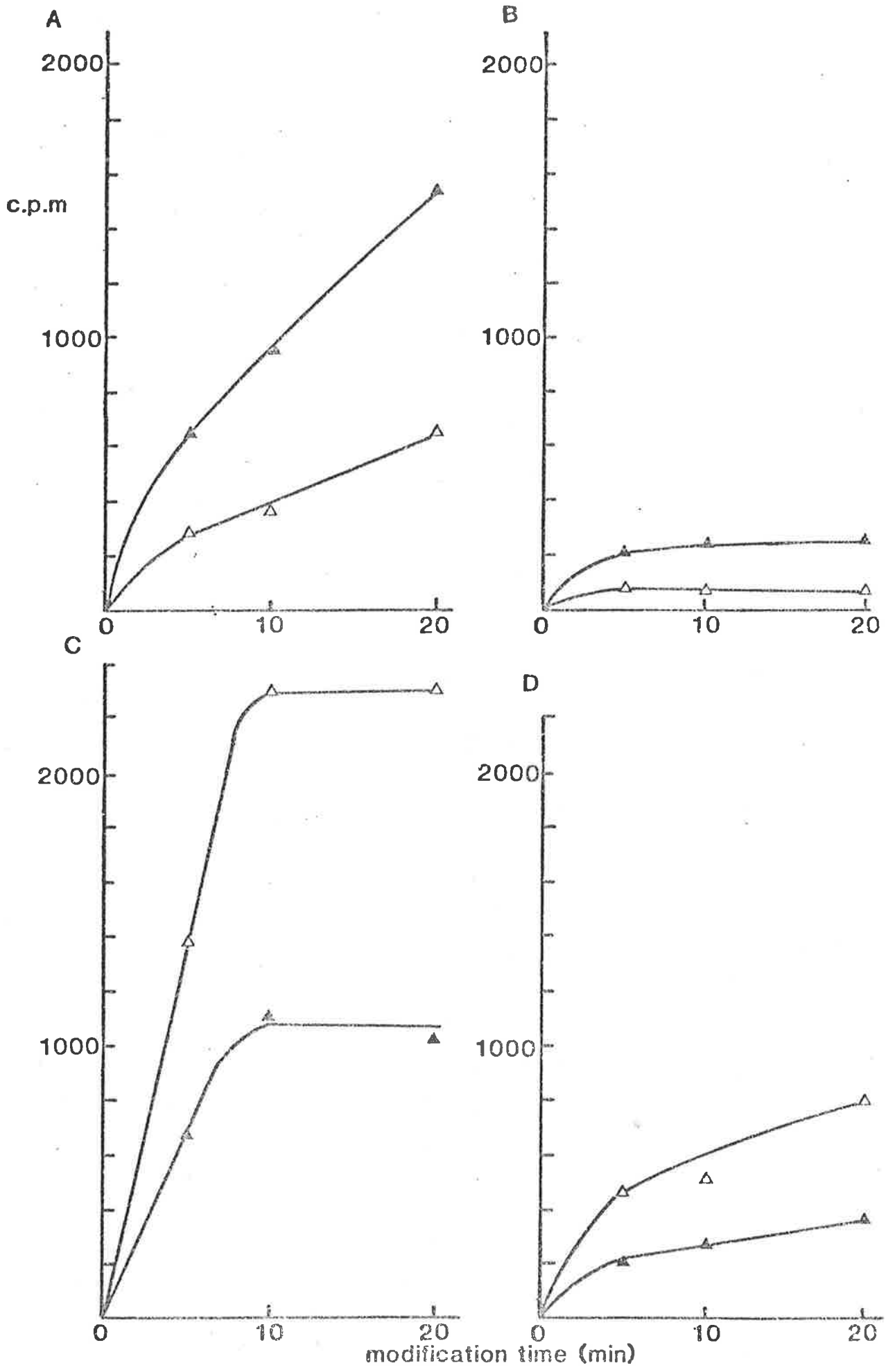

A
B
C
D
2000
1000
c.p.m
0
10
20
modification time (min)

FIGURE 4.6

INHIBITION OF PROPIONYL-CoA CARBOXYLASE BY oATP

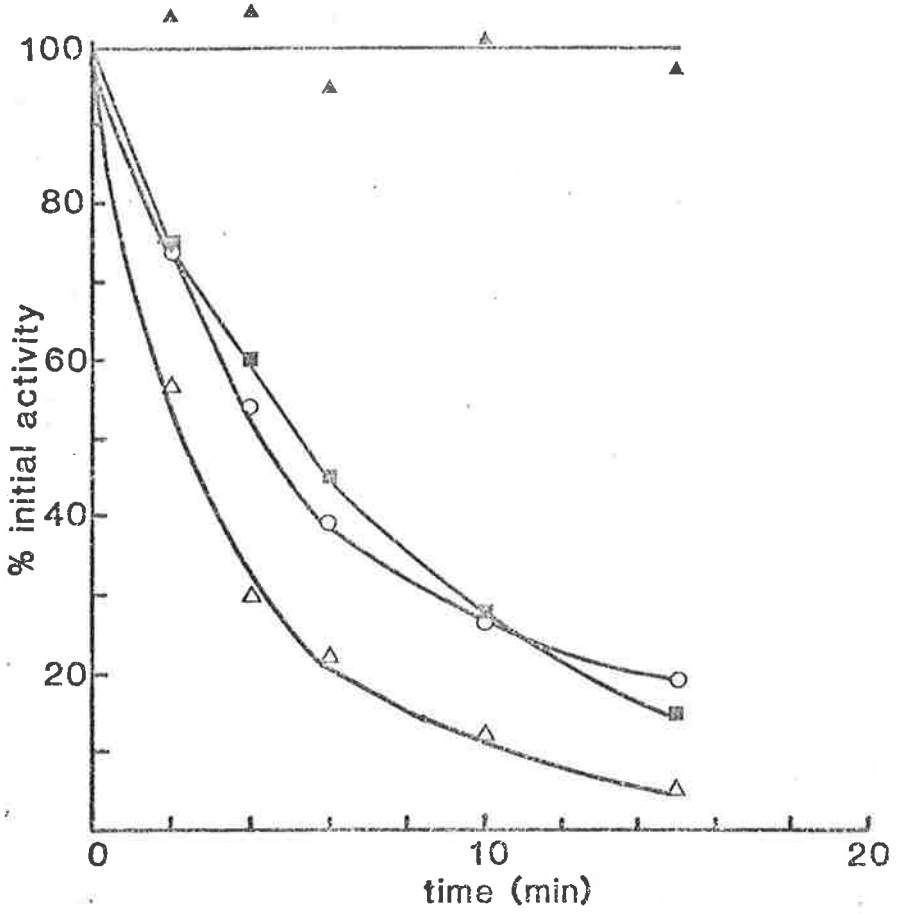
The inactivation mixture contained propionyl-CoA carboxylase, 10.6 units/ml; 0.1 M N-ethylmorpholine-acetate pH 8.0, 10 mM $MgCl_2$ and 1 mM oATP, except for the control (▲), which contained no oATP. Additions were 1.1 mM propionyl-CoA (o), 1 mM MgATP (■), 10 mM $NaHCO_3$ (Δ).

The mixture was incubated at 25°C and 15 µl aliquots removed at the designated times and assayed for enzymic activity.

A. % initial activity versus time

B. log (% initial activity) versus time

a.


100
80
60
40
20
% initial activity
0
10
20
time (min)

b.

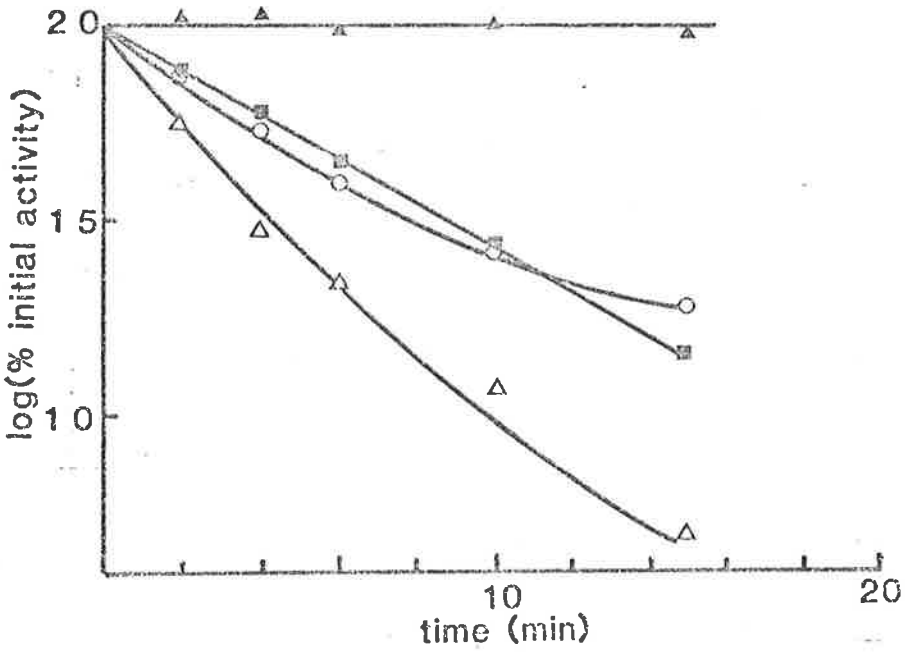

20
15
10
log(% initial activity)
10
20
time (min)

FIGURE 4.7

INHIBITION OF PROPIONYL-CoA CARBOXYLASE BY oATP

The inactivation mixture contained 100 mM N-ethylmorpholine-acetate, pH 8.0, 10 mM $MgCl_2$, 2.6 units/ml propionyl-CoA carboxylase and where present 0.22 mM oATP. Additions were 1 mM ATP (□), 0.8 mM propionyl-CoA (○), 1 mM ATP and 0.8 mM propionyl-CoA (△) and no additions (■). The control (▲) did not contain oATP. The mixture was incubated at 25°C and at the designated times aliquots of 15 µl were removed and assayed for enzymic activity.

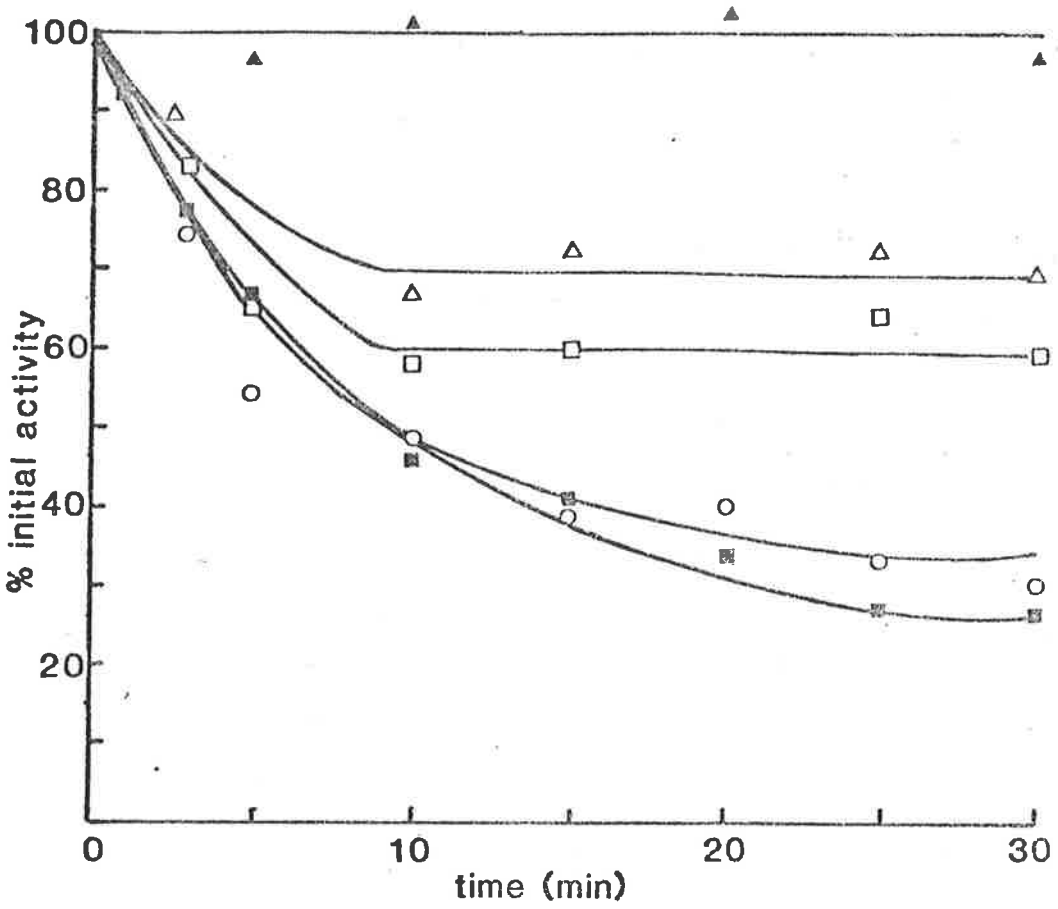

100
80
60
40
20
% initial activity
0
10
20
30
time (min)

FIGURE 4.8

COVALENT MODIFICATION OF THE SUBUNITS OF PROPIONYL-CoA CARBOXYLASE BY oATP

The enzyme was modified with 0.5 mM o[^{14}C]ATP as described in Section 4.2.5 and electrophoresed in SDS-polyacrylamide tube gels. The radioactivity in each 1 mm gel slice has been standardized with respect to total protein in the gel. The relative peak areas are shown as percentages.

A. No additions

B. 15 mM Mgpropionyl-CoA included

C. 15 mM MgATP included

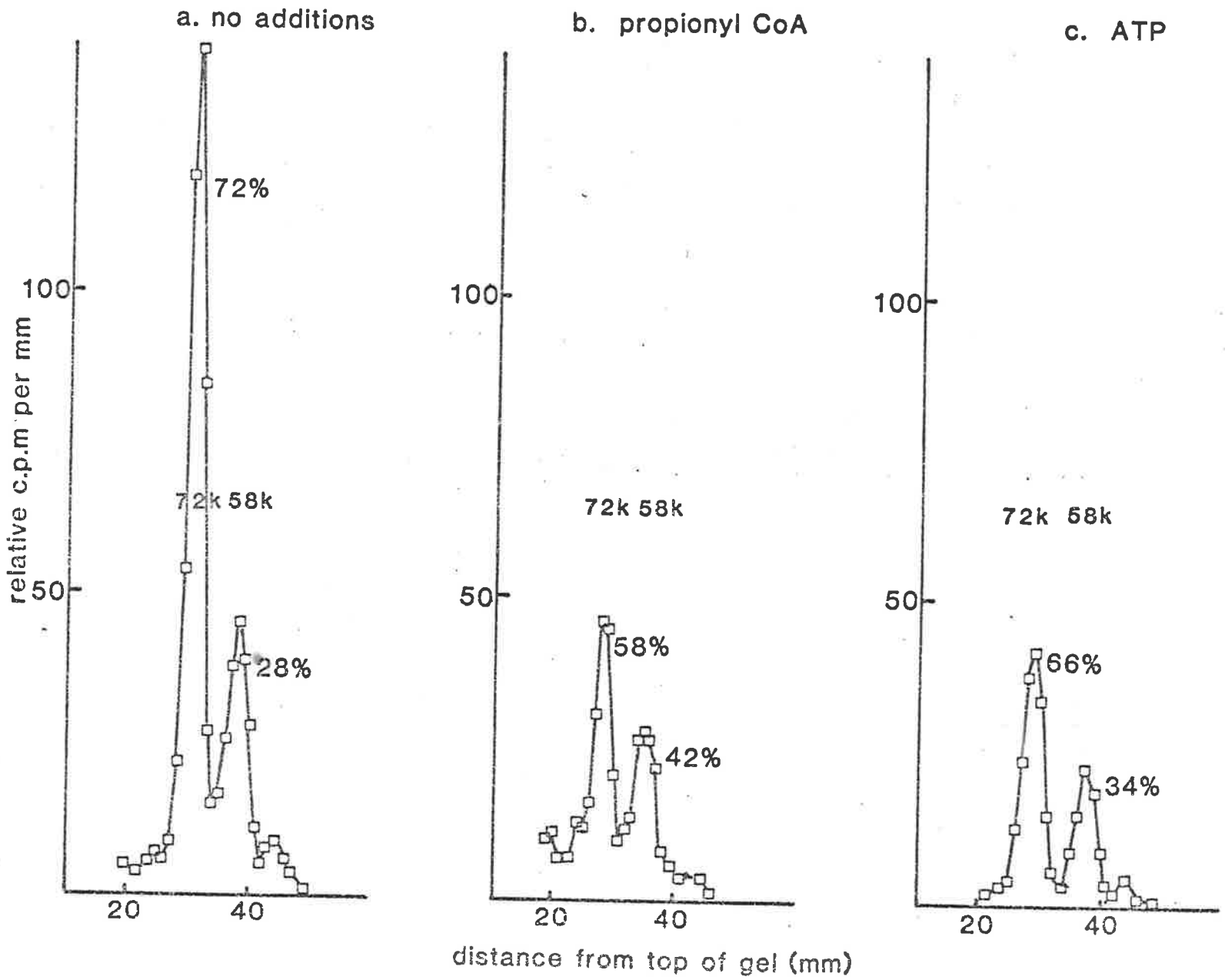

a. no additions
b. propionyl CoA
c. ATP
relative c.p.m per mm
100
50
72%
28%
72k 58k
58%
42%
66%
34%
20
40
distance from top of gel (mm)

FIGURE 4.9

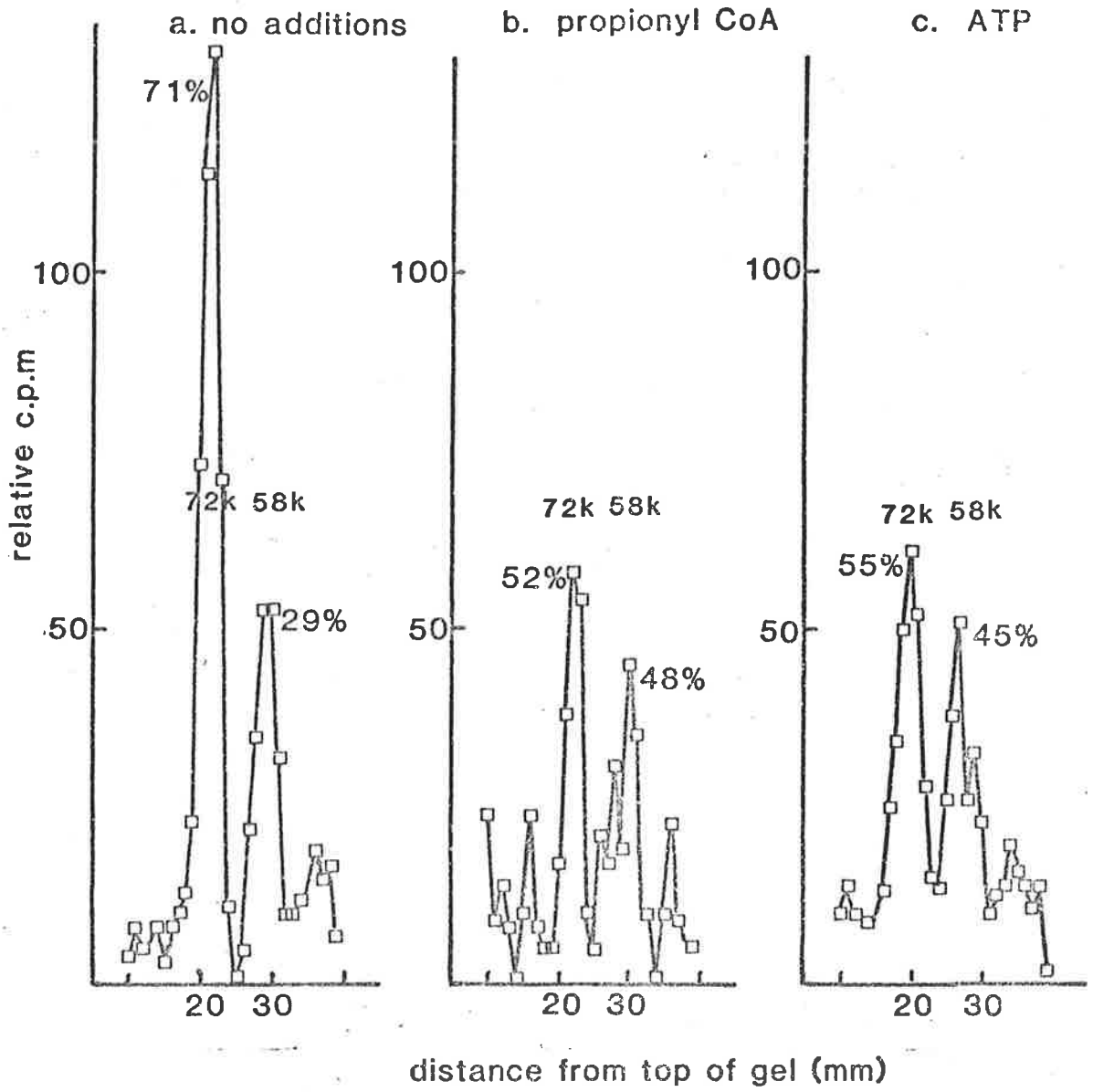
COVALENT MODIFICATION OF THE SUBUNITS OF PROPIONYL-CoA CARBOXYLASE BY o-DEPHOSPHO-PROPIONYL-CoA

The conditions of the experiment were the same as for Fig. 4.8 except that 0.65 mM o-dephosphopropionyl-[^{3}H]CoA (76,000 cpm/nmole) was used instead of oATP.

A. no additions

B. 15 mM Mgpropionyl-CoA included

C. 15 mM MgATP included


a. no additions
b. propionyl CoA
c. ATP
71%
72k 58k
29%
52%
48%
55%
45%
100
50
relative c.p.m
20 30
distance from top of gel (mm)

CHAPTER 5

COMPARISONS OF THE AMINO ACID COMPOSITIONS OF BIOTIN-DEPENDENT ENZYMES

5.1 INTRODUCTION

While it is tempting to speculate (as I have in Section 4.1) that some biotin-dependent enzymes have evolved from a common ancestral protein, there is little available evidence that these enzymes do in fact constitute an evolutionary family, apart from some sequence homology at the biotin attachment point. (The evidence has been discussed in Section 1.1.4). One method which has not been employed in addressing this problem is the comparison of amino acid compositions, despite the accumulation of a substantial data base. As pointed out by Cornish-Bowden (1980a), comparisons of amino acid compositions can be almost as reliable as sequence comparisons, and are much easier to obtain. In this study, the amino acid compositions of 21 biotin-containing proteins and related subunits have been used to gain some insight into the relationships between the biotin enzymes.

5.2 METHODS FOR COMPARING AMINO ACID COMPOSITIONS

5.2.1 The SΔn index

A computer program, shown at the end of this chapter, was written to calculate the index SΔn, i.e.,

$$S\Delta n = \tfrac{1}{2} \Sigma(n_{iA} - n_{iB})^2 - 0.035\ (N_A - N_B)^2 + 0.535|N_A - N_B|$$

where n_{iA} and n_{iB} are the numbers of amino acid residues of the ith type in proteins A and B of lengths N_A and N_B respectively. The first term [$\tfrac{1}{2} \Sigma(n_{iA} - n_{iB})^2$] is an unbiased estimator of the number of differences between two protein sequences of equal length (Cornish-Bowden, 1977). The last

two terms are approximate correction terms to allow for differences in length (Cornish-Bowden, 1979).

5.2.2 The correlation co-efficient

Since the amino acid compositions of proteins as a whole are correlated, determination of a correlation co-efficient directly on the figures for residues per hundred would be difficult to interpret. Transforming the data to percentile values overcomes this problem (Reisner & Westwood, 1977). Reeck (1976) has compiled tables of the frequency of occurrence of each amino acid in a set of 207 mutually non-homologous proteins. Reeck's data is shown as a percentile chart in Table 5.1. To compare the amino acid compositions of two proteins, the mole % value for each amino acid of each protein was first converted to a percentile value using Table 5.1 and interpolating linearly. A correlation co-efficient was then calculated as follows:

$$r = \frac{\Sigma\, x_i\, y_i}{\sqrt{\Sigma\, x_i^2 \;\; \Sigma\, y_i^2}}$$

where x_i and y_i are the percentile values for amino acid i in proteins X and Y respectively.

Computer programs for calculating percentile values and correlation co-efficients are shown at the end of this chapter.

5.2.3 Construction of phylogenetic trees

The correlation co-efficient was used as a measure of the time since divergence. The tree was constructed by an adaption of the unweighted pair-group method of clustering described by Sokal & Sneath (1963). A matrix of correlation co-efficients among the proteins under consideration was constructed and the most closely related pair of proteins identified. The amino acid compositions of these two proteins were pooled by averaging the mole % values and the average composition was taken to represent the ancestor at the time of divergence. A new matrix of correlation co-efficients was calculated, to find the next most closely related pair of proteins, and so on, until all the proteins had been pooled. When pooling two different sized groups of proteins, the "ancestral" composition was taken as the arithmetic mean of the mole % values of all of the comprised proteins.

A computer program written to carry out these operations is shown at the end of this chapter.

5.3 RESULTS

5.3.1 Comparisons using the SΔn index

When SΔn is divided by the length of the longer protein, SΔn/N represents an estimate of the proportion of the amino acid sequence of the longer protein that is different from the amino acid sequence of the shorter protein. Cornish-Bowden (1980b) has suggested critical values for testing the significance of SΔn/N. SΔn/N values should exceed 0.42 in approximately 95% of comparisons between pairs of proteins

that have the same statistical properties but are otherwise unrelated. Thus a value of $S\Delta n/N$ less than 0.42 is a strong indication that two proteins are related. On the other hand, if the $S\Delta n/N$ value is greater than 0.93, it is unlikely the proteins are related.

The amino acid compositions of each of the 21 biotin-dependent enzymes or subunits included in this study are shown in Table 5.2. $S\Delta n/N$ values were calculated for each pair of proteins whose lengths differed by less than 32 residues. The errors involved in correcting for length differences prohibit the use of the $S\Delta n$ index where the length difference is large. The results obtained are shown in Table 5.3. Of the 32 comparisons made, 6 have $S\Delta n/N$ values less than 0.42, indicating that the following proteins are very likely related: sheep kidney pyruvate carboxylase with sheep liver, rat liver, and guinea pig liver pyruvate carboxylases; sheep liver with rat liver pyruvate carboxylase; rat with chicken acetyl-CoA carboxylase; the 2.5 S_H with the 2.5 S_E subunit of transcarboxylase. In a further eight comparisons the value of $S\Delta n/N$ was less than 0.93, indicating possible relationships.

Relationships between the same protein in different species are to be expected and are not of interest here, except as a pre-requisite for detecting relationships between different proteins in different species. Of the 9 comparisons between the acetyl-CoA carboxylases and the pyruvate carboxylases, only 3 yielded $S\Delta n/N$ values less than 0.93. This suggests that acetyl-CoA carboxylase and pyruvate carboxylase are not related. Nothing can be said about relation-

ships between the other proteins as their differences in length are too great to use the $S\Delta n$ index. Although Cornish-Bowden (1980b) questions the usefulness of comparing the compositions of proteins of unknown sequence and appreciably different size, when dealing with proteins of about 1000 residues it does not seem unreasonable to compare proteins whose lengths differ by up to 100 residues.

5.3.2 Comparisons using the correlation co-efficient

The advantages of using the correlation co-efficient as an index of protein relatedness are firstly, the critical values for various levels of significance are known (Fisher & Yates, 1963), and secondly, the method can be applied to proteins of different length.

The amino acid compositions of the 21 proteins shown in Table 5.2 were compared pair-wise and the resulting correlation co-efficients are shown in Table 5.4. Tryptophan was not included in the calculations, so the correlation co-efficients have 15 degrees of freedom. Also shown in Table 5.4 are the values of the correlation co-efficient for different levels of significance (Fisher & Yates, 1963). For comparison, Table 5.5 shows the results of such an analysis on the serine proteases, a group of proteins classified as a family on the basis of amino acid sequence (Dayhoff & Hunt, 1972). Sheep liver pyruvate carboxylase was included as a "control". If initially only correlations significant at the 2% level are considered the biotin-related proteins fall into five groups (see Fig. 5.1). The groups are:

(a) sheep liver propionyl-CoA carboxylase and its subunits,

pig heart propionyl-CoA carboxylase, and the "acyl-CoA carboxylase" of the free-living nematode *Turbatrix aceti*

(b) acetyl-CoA carboxylases from chicken, rat and rabbit

(c) β-methylcrotonyl-CoA carboxylase from *Achromobacter* and pyruvate carboxylases from sheep kidney, sheep liver, chicken liver, pigeon liver, rat liver and guinea pig liver

(d) pyruvate carboxylase from *Pseudomonas citronellolis* and its subunits

(e) transcarboxylase from *Propionibacterium shermanii* and its subunits.

The only inter-group correlations significant at the 2% level are between β-methylcrotonyl-CoA carboxylase and the 2.5 S_H subunit of transcarboxylase ($P < 1\%$) and rat liver pyruvate carboxylase and each of the three acetyl-CoA carboxylases ($P < 0.02$ for each).

The following correlations become significant at the 5% level:

- sheep liver propionyl-CoA carboxylase and rat liver acetyl-CoA carboxylase
- rabbit acetyl-CoA carboxylase and each of sheep kidney, sheep liver and chicken liver pyruvate carboxylase
- the 54k subunit of *P. citronellolis* pyruvate carboxylase and each of pigeon and guinea pig pyruvate carboxylase
- chicken pyruvate carboxylase and *P. citronellolis*

pyruvate carboxylase

- β-methylcrotonyl-CoA carboxylase and each of *P. citronellolis* pyruvate carboxylase and its 54k subunit
- β-methylcrotonyl-CoA carboxylase and the 2.5 S_E subunit of transcarboxylase (and also the total transcarboxylase).

These inter-group correlations are shown schematically in Table 5.4. Also shown are the correlations significant at the 10% level. At this rather weak level of significance there are five correlations between the transcarboxylase group and the pyruvate carboxylase group.

5.3.3 Phylogenetic tree

The 21 biotin-related proteins shown in Table 5.2 were used to construct a phylogenetic tree, using the method described in Section 5.2.3. The tree obtained (Fig. 5.2) indicates β-methylcrotonyl-CoA carboxylase diverged from pyruvate carboxylase relatively recently. The other correlation co-efficients between the different "ancestral" biotin-dependent enzymes are too small to be of significance. Thus although a tree can be constructed, showing propionyl-CoA carboxylase diverging from the rest early, and pyruvate carboxylase diverging from transcarboxylase more recently, the amino acid compositions provide no evidence that they are in fact related.

5.4 DISCUSSION

Various indexes have been used to express the amount of difference between amino acid compositions (Metzger *et al.*, 1968; Harris *et al.*, 1969; Marchalonis & Weltman, 1971; Harris & Teller, 1973). However, none of these indexes have a rigorous statistical basis and so it is difficult to test the significance of and draw conclusions from the values obtained. To overcome this problem, Cornish-Bowden (1977) has devised an index based on a theoretical framework that allows tests of significance to be applied (Cornish-Bowden, 1980b). The method of Cornish-Bowden, however, has the drawback that it can only be applied to proteins of nearly identical length.

By transforming amino acid composition figures to percentile values, we can apply the correlation co-efficient as an index of relatedness (Reisner & Westwood, 1977). Significance levels of the correlation co-efficient are known (Fisher & Yates, 1963) and the method can be applied to proteins of rather different lengths.

The validity of the correlation co-efficient as an index of protein relatedness relies on the assumption that the general occurrence of each amino acid in proteins, when expressed in terms of mole %, has a normal distribution. As several amino acids (in particular *pro*, *cys*, *glu* and *gly*) deviate somewhat from being normally distributed (Reeck & Fisher, 1973), the levels of significance are not exact, but are approximations. It should also be borne in mind that strong correlations do not force the conclusion of

common ancestry. However, this is the most likely cause of similarities in amino acid compositions. One possible alternative cause is convergent evolution, but this is more likely to occur to special regions of proteins, rather than whole molecules.

5.4.1 Close relationships

The "acyl-CoA carboxylase" isolated from the free living nematode, *Turbatrix aceti*, by Meyer *et al.* (1978) correlates quite strongly with the propionyl-CoA carboxylases and not at all with the acetyl-CoA carboxylases. This is not surprising as its kinetic properties (Meyer & Meyer, 1978) and the size and number of its subunits (Meyer *et al.*, 1978) are much more like those of propionyl-CoA carboxylase than acetyl-CoA carboxylase.

It is curious that β-methylcrotonyl-CoA carboxylase from *Achromobacter* correlates strongly with the vertebrate pyruvate carboxylases while *Pseudomonas* pyruvate carboxylase correlates much less strongly with the vertebrate pyruvate carboxylases. It seems very likely that at least β-methylcrotonyl-CoA carboxylase and pyruvate carboxylase share an evolutionary ancestor. It is perhaps significant that the vertebrate pyruvate carboxylases and β-methylcrotonyl-CoA carboxylase have an acyl-CoA binding site while *Pseudomonas citronellolis* pyruvate carboxylase apparently does not (Taylor *et al.*, 1975).

The comparisons made here do not support the rather appealing hypothesis that some of the biotin-dependent enzymes have commonly derived subunits; for example, the

pyruvate-binding subunits of transcarboxylase and *Pseudomonas* pyruvate carboxylase, the propionyl-CoA-binding subunits of transcarboxylase and propionyl-CoA carboxylase, or the ATP-binding subunits of propionyl-CoA carboxylase and *Pseudomonas* pyruvate carboxylase. None of the inter-enzyme subunit comparisons yield significant correlation co-efficients.

On the other hand, in each of the three enzymes whose subunits have been analysed, the different subunits of the same enzyme correlated strongly, suggesting each pair has arisen from a gene duplication. This fits with the work of Berger & Wood (1976) who showed that the two larger subunits of transcarboxylase are immunologically cross-reactive. It also fits with one of the possible conclusions from the affinity labelling studies on propionyl-CoA carboxylase, described in Chapter 4, i.e., that both subunits contribute to the binding of ATP and propionyl-CoA.

5.4.2 Weaker correlations

The averaging procedure used in constructing the phylogenetic tree shown in Fig. 5.2 would tend to increase the likelihood of obtaining higher correlations by chance alone, as larger and larger groups of proteins are compared. Even so, there is no indication that any of the biotin-dependent enzymes arose from a common ancestor, apart from pyruvate carboxylase and β-methylcrotonyl-CoA carboxylase. However, when the correlations between individual proteins are examined (Table 5.4), we find that of the 18 correlations between the acetyl-CoA carboxylases and the vertebrate

pyruvate carboxylases, 6 are significant at the 5% level. The correlation between β-methylcrotonyl-CoA and trans-carboxylase is also significant at the 5% level. Thus we cannot dismiss the idea that the biotin-dependent enzymes constitute a protein family. It would be worthwhile to re-examine the amino acid compositions of these enzymes when more biotin-dependent enzymes have been subjected to amino acid analysis.

TABLE 5.1 MOLE % DISTRIBUTIONS OF AMINO ACID RESIDUES IN THE REECK SET OF PROTEINS AT 5 PERCENTILE INTERVALS (REECK, 1976)

	PERCENTILE																		
	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
RESIDUE	MOLE % VALUES																			
LYS	2.4	3.3	4.0	4.4	4.7	5.0	5.2	5.5	5.8	6.1	6.6	6.9	7.2	7.5	7.7	8.1	8.6	9.7	10.8	17.3
HIS	.0	.8	1.1	1.3	1.5	1.7	1.8	1.9	2.0	2.1	2.3	2.4	2.5	2.6	2.9	3.1	3.2	3.4	4.1	7.1
ARG	1.3	2.0	2.7	2.9	3.1	3.4	3.6	3.8	4.0	4.3	4.5	4.7	5.0	5.2	5.4	5.7	6.2	6.6	7.4	13.7
ASX	6.8	7.6	7.9	8.6	9.1	9.4	9.7	10.0	10.3	10.6	10.9	11.2	11.3	11.6	11.8	12.4	13.0	13.9	15.2	19.2
THR	3.0	3.6	3.9	4.4	4.6	4.9	5.0	5.1	5.3	5.4	5.6	5.8	5.9	6.4	6.7	7.0	7.3	7.9	8.7	15.0
SER	2.4	3.7	4.1	4.5	4.7	5.0	5.2	5.4	5.7	5.8	6.1	6.3	6.7	6.9	7.8	8.2	8.5	9.7	11.5	65.5
GLX	5.8	7.2	7.8	8.5	8.8	9.0	9.2	9.6	9.8	10.1	10.4	10.8	11.0	11.5	11.8	12.3	13.3	14.2	16.7	24.8
PRO	1.8	3.0	3.3	3.6	3.8	3.9	4.1	4.3	4.4	4.6	4.7	4.9	5.0	5.2	5.5	5.7	6.2	6.9	7.8	22.1
GLY	3.8	4.8	5.3	6.1	6.6	6.9	7.1	7.4	7.7	8.0	8.2	8.5	8.6	9.0	9.3	9.7	10.1	10.9	12.5	34.0
ALA	3.9	5.4	5.8	6.3	6.6	7.1	7.6	7.9	8.2	8.4	8.6	9.0	9.2	9.6	9.8	10.1	10.7	11.5	13.5	17.2
CYS	.0	.0	.5	.7	.9	.9	1.0	1.2	1.3	1.5	1.6	1.7	2.0	2.2	2.6	3.1	3.9	5.8	8.6	15.2
VAL	2.9	4.5	5.1	5.4	5.8	6.0	6.2	6.4	6.6	6.7	6.9	7.2	7.4	7.7	7.9	8.1	8.5	9.2	10.2	11.5
MET	.0	.4	.7	.9	1.0	1.2	1.4	1.5	1.7	1.8	2.0	2.1	2.3	2.6	2.7	2.9	3.0	3.2	3.6	5.0
ILE	1.9	2.6	3.1	3.5	3.9	4.1	4.3	4.6	4.7	5.0	5.3	5.4	5.7	6.0	6.1	6.3	6.6	7.1	7.7	8.5
LEU	3.9	5.3	5.9	6.2	6.6	6.9	7.1	7.5	7.8	8.0	8.2	8.6	8.9	9.2	9.6	9.9	10.5	11.2	12.0	15.5
TYR	1.1	1.6	1.8	2.0	2.3	2.4	2.6	2.8	3.0	3.1	3.2	3.4	3.6	3.8	4.0	4.3	4.7	5.0	6.0	10.9
PHE	1.3	2.0	2.3	2.6	2.9	3.0	3.2	3.4	3.6	3.7	3.8	4.1	4.2	4.4	4.6	4.8	5.0	5.3	5.9	8.1

TABLE 5.2 AMINO ACID COMPOSITIONS OF VARIOUS BIOTIN-DEPENDENT ENZYMES AND THEIR SUBUNITS.

Abbreviation	Protein	Reference
SLPCC	Sheep liver propionyl CoA carboxylase	This thesis
SLPCC72	" 72,000 MW subunit	This thesis
SLPCC58	" 58,000 MW subunit	This thesis
PHPCC	Pig heart propionyl CoA carboxylase	Kaziro *et al.* (1965)
P.CIT PC	*Pseudomonas citronellolis* pyruvate carboxylase	Duc (1981)
P.CIT A	" 65,000 MW subunit	Duc (1981)
P.CIT B	" 54,000 MW subunit	Duc (1981)
SKPC	Sheep kidney pyruvate carboxylase	Bais (1974)
SLPC	Sheep liver pyruvate carboxylase	Bais (1974)
CLPC	Chicken liver pyruvate carboxylase*	Bais (1974)
PLPC	Pigeon liver pyruvate carboxylase	Bais (1974)
GPLPC	Guinea Pig liver pyruvate carboxylase	Bais (1974)
RLPC	Rat liver pyruvate carboxylase	Bais (1974)
T.ACETI	*Turbatrix aceti* acyl CoA carboxylase	Meyer *et al.* (1978)
RABBIT ACC	Rabbit acetyl CoA carboxylase	Hardie & Cohen (1978)
RLACC	Rat liver acetyl CoA carboxylase	Inoue & Lowenstein (1972)
CLACC	Chicken liver acetyl CoA carboxylase	Gregolin *et al.* (1966)
BMCCC	β-methylcrotonyl CoA carboxylase	Apitz-Castro *et al.* (1970)
TC2.5 SH	Transcarboxylase 2.5 S_H subunit	Wood & Zwolinski (1976)
TC2.5 SE	Transcarboxylase 2.5 S_E subunit	Wood & Zwolinski (1976)
TC	*Propionibacterium shermanii* transcarboxylase	Wood & Zwolinski (1976)

* The analysis of chicken liver pyruvate carboxylase published by Scrutton & Utter (1965b) was not used as it has since been shown by Goss *et al.* (1979) and Cohen *et al.* (1979b) that enzyme prepared by the method of Scrutton & Utter is impure.

AMINO ACID COMPOSITIONS IN MOLE %

RESIDUE	LYS	HIS	ARG	ASX	THR	SER	GLX	PRO	GLY	ALA	CYS	VAL	MET	ILE	LEU	TYR	PHE
SLPCC	5.8	3.2	6.5	8.2	5.2	6.3	6.5	5.3	7.9	7.8	2.0	7.0	3.0	6.1	7.1	3.8	5.2
SLPCC72	6.5	3.9	6.0	9.2	5.8	7.3	8.6	3.8	8.8	8.9	.7	6.8	3.5	6.8	7.5	.8	5.1
SLPCC58	5.1	3.7	5.4	11.2	5.9	6.4	7.9	5.8	8.8	8.5	.5	9.0	3.2	5.2	6.4	2.3	4.9
FHPCC	6.0	3.2	5.7	11.3	5.1	6.8	3.9	5.2	8.9	9.9	1.6	8.5	2.8	6.7	7.5	2.8	4.5
T.ACETI	6.5	2.3	4.8	11.0	5.4	5.9	9.6	4.4	8.4	9.9	1.0	8.8	2.0	5.9	7.1	2.9	4.1
RABBITACC	4.3	2.5	5.7	9.4	4.6	7.0	13.0	5.3	6.8	7.5	2.0	7.6	2.2	5.2	9.8	3.0	3.8
RLACC	5.1	2.9	6.2	9.3	5.0	6.2	12.0	4.9	6.3	6.8	1.5	8.2	3.2	5.6	9.2	3.4	4.2
CLACC	4.8	2.6	6.5	10.2	4.4	5.1	11.9	5.1	6.7	7.0	1.2	8.1	3.2	6.4	9.2	3.5	4.1
P.CITPC	4.7	2.0	4.2	7.3	5.2	4.5	10.5	.8	19.2	9.9	1.2	6.8	2.0	5.1	8.0	2.7	3.3
P.CITA	5.5	1.8	4.8	8.4	5.3	7.3	10.4	2.4	18.5	9.8	1.2	6.4	1.5	4.6	7.9	2.4	2.8
P.CITB	5.1	1.8	6.4	8.2	5.1	6.8	12.7	2.2	14.0	10.0	1.2	5.3	1.5	5.8	7.3	2.7	2.9
SKPC	4.9	2.9	5.0	6.4	5.3	6.3	11.8	5.6	9.3	8.8	1.1	7.3	2.0	5.3	8.9	2.7	4.0
SLPC	5.1	2.9	5.5	9.0	5.9	5.9	11.3	5.5	8.9	8.8	1.2	7.1	2.1	5.3	8.4	3.0	4.1
CLPC	3.6	2.6	6.4	7.5	4.1	5.2	11.5	6.4	10.5	11.0	1.1	7.8	1.7	4.2	9.8	2.3	3.9
PLPC	3.8	1.7	6.8	9.7	4.2	5.2	12.4	6.6	10.4	10.7	1.1	6.7	1.5	4.2	8.3	2.4	3.4
GPLPC	5.3	2.6	5.0	8.9	5.5	6.4	14.1	5.1	10.2	8.3	1.1	6.4	1.7	5.0	7.7	2.6	4.3
RLPC	5.1	3.1	5.4	8.7	5.2	5.8	11.9	5.7	9.0	9.0	1.1	7.2	2.6	5.3	8.9	2.9	4.3
BMCCC	2.9	2.4	6.2	10.1	4.8	5.3	12.8	4.8	8.9	12.0	1.1	7.0	2.2	5.2	8.4	2.4	3.3
TC2.5SH	5.4	1.6	4.3	11.1	4.9	4.5	12.9	4.9	9.1	11.2	1.3	7.6	3.6	4.9	6.7	2.0	4.0
TC2.5SE	6.3	2.2	4.8	9.8	5.5	4.4	13.4	6.1	7.6	10.3	.8	7.4	5.2	3.5	6.8	2.9	2.9
TC	6.2	1.8	4.3	10.2	5.4	4.2	12.8	5.4	9.2	10.8	1.0	7.9	4.3	4.3	6.7	2.3	3.2

TABLE 5.3

COMPARISONS USING THE $S\Delta n/N$ INDEX

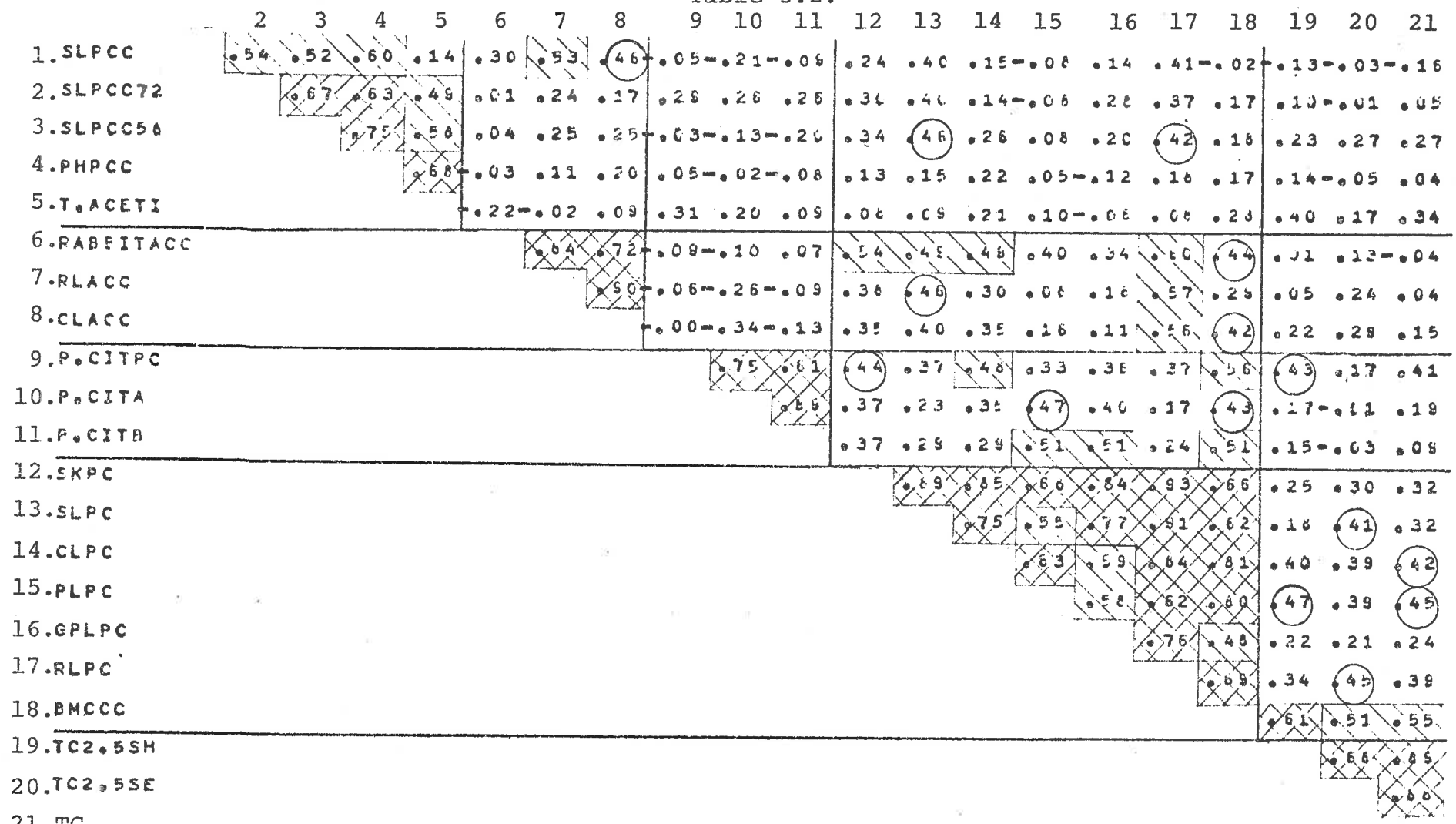
PROTEIN	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. SLPCC																				
2. SLPCC72																				
3. SLPCC58									>1									>1	>1	
4. PHPCC				>1			>1													
5. T.ACETI				>1																
6. RABBITACC																				
7. RLACC							.19				>1	.84	>1		>1	.81				
8. CLACC											>1	>1	>1		>1	.86				
9. P.CITPC														>1				>1	>1	
10. P.CITA																				
11. P.CITB																				
12. SKPC												.16	.78	.91	42	.12				
13. SLPC															.54	.09				
14. CLPC																.93				
15. PLPC																				
16. GPLPC																.54				
17. RLPC																				
18. BMCCC																				
19. TC2.5SH																			.42	
20. TC2.5SE																				
21. TC																				

The protein abbreviations are the same as those used in Table 5.2

$S\Delta n/N$ < 0.42 indicates that the proteins are probably related.
$S\Delta n/N$ > 1 indicates that the proteins are probably not related.

TABLE 5.4 COMPARISONS USING THE CORRELATION CO-EFFICIENT AS AN INDEX

The protein names are given in full in the legend to Table 5.2.



LEVELS OF SIGNIFICANCE FOR 15 DEGREES OF FREEDOM

P	0.1	0.05	0.02	0.01	0.001
r	.4124	.4821	.5577	.6055	.7246
Significance coding	(43)	[53]		[63]	

TABLE 5.5 CORRELATION CO-EFFICIENT COMPARISONS OF THE COMPOSITIONS OF SERINE PROTEASES AND SHEEP LIVER PYRUVATE CARBOXYLASE.

	2	3	4	5	6	7	8
1 BOV.TYRPSN	.8406	.7073	.6282	.7457	.6852	.5698	.1720
2 DOGFISHTRP	-I	.6110	.6620	.8112	.7964	.6693	.1120
3 CHYMO.A	-I	-I	.9639	.7542	.5064	.3945	.1426
4 CHYMO.B	-I	-I	-I	.7647	.5547	.3643	.1054
5 ELASTASE	-I	-I	-I	-I	.7725	.6563	.2579
6 THROMBIN.B	-I	-I	-I	-I	-I	.5310	.4619
ALPHALYTIC	-I	-I	-I	-I	-I	-I	.1562

The compositions of the serine proteases were obtained from Dayhoff & Hunt (1972). The composition of sheep liver pyruvate carboxylase is from Bais (1974).

1. trypsinogen from cattle
2. trypsinogen from spiny dogfish
3. chymotrypsinogen A chain from cattle
4. chymotrypsinogen B chain from cattle
5. elastase from pig
6. thrombin B chain from cattle
7. α-lytic protease from *Myxobacter*
8. pyruvate carboxylase from sheep liver

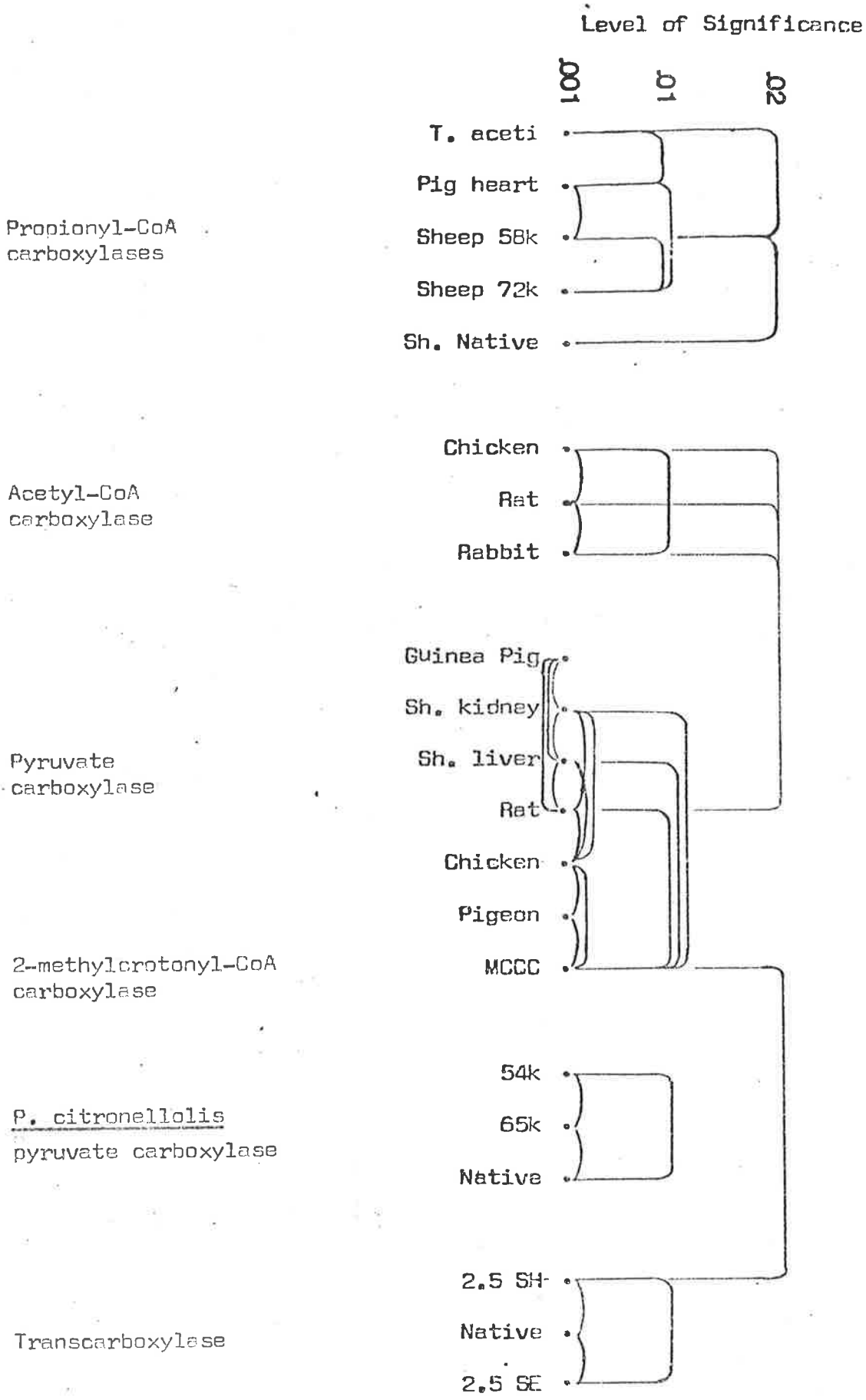


Fig. 5.1 Correlations significant at the 2% level.

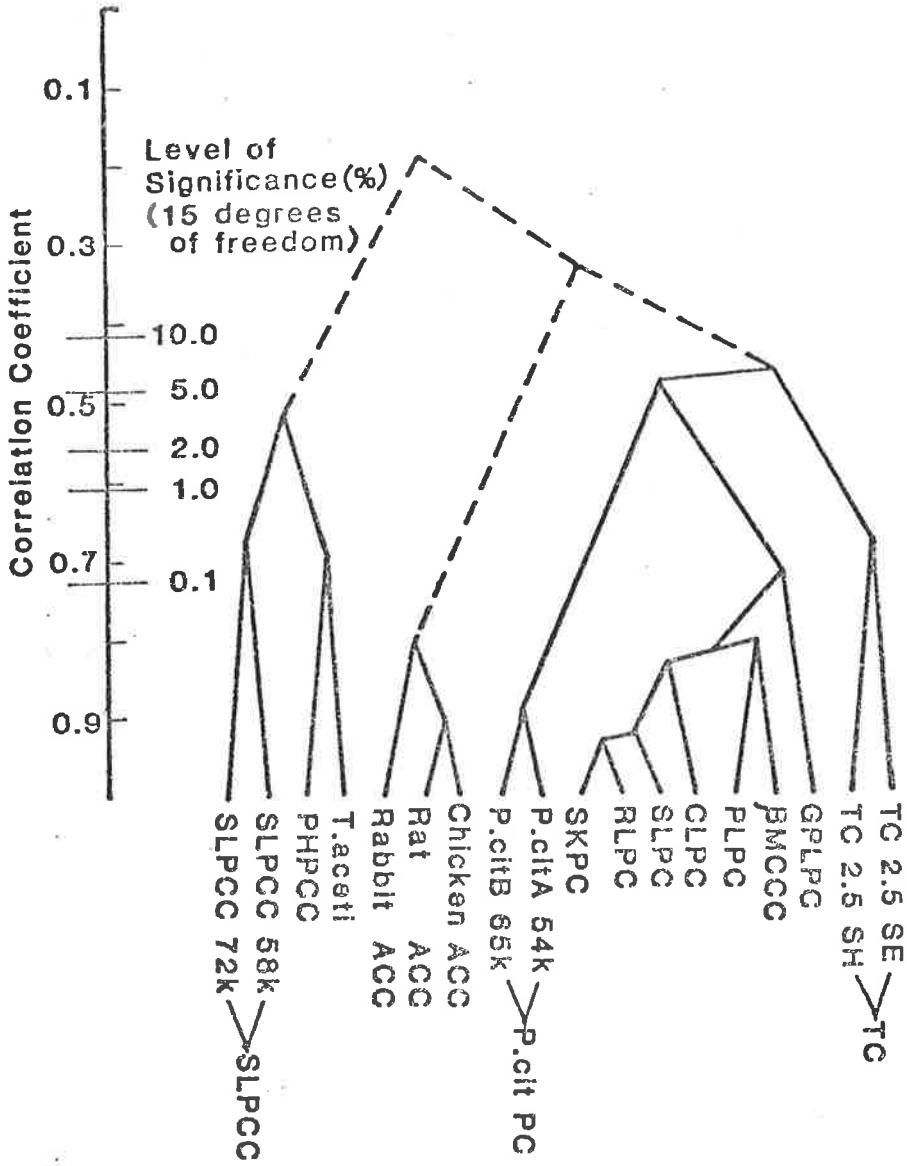


FIGURE 5.2

PHYLOGENETIC TREE FOR BIOTIN-DEPENDENT ENZYMES

The correlation co-efficient was used as an index for comparing amino acid compositions. The phylogenetic tree was constructed as described in Section 5.2.3. After the subunits of an enzyme were compared, the composition of the whole enzyme was used rather than the average of the subunits. Protein names are given in full in the legend to Table 5.2.

```
COMPARISONS USING SΔN/N AS AN INDEX
C
C
      PROGRAM CB(INPUT,OUTPUT,TAPE5=/80,TAPE8)
      DIMENSION AA(20,17),NAME(20),DIF2(17)
      REWIND 5
      DO 20 I=1,20
      READ(5,*) NAME(I),(AA(I,J),J=1,17)
      IF(EOF(5))30,20
 20   CONTINUE
 30   PRINT 1000
 1000 FORMAT(,/,* WHICH PROTEINS DO YOU WANT TO COMPARE↓*,/,)
      READ*,NPA,NPB
      IF(NPA.EQ.0)GO TO 99
      SS=XNA=XNB=0
      DO 40 J=1,17
      DIF2(J)=(AA(NPA,J)-AA(NPB,J))**2
 40   SS=SS+DIF2(J)
      DO 50 J=1,17
      XNA=XNA+AA(NPA,U)
 50   XNB=XNB+AA(NPB,J)
      CFAC=.535*ABS(XNA-XNB)-.035*(XNA-XNB)**2
      SN=SS/2+CFAC
      XN=AMAX1(XNA,XNB)
      SNN=SN/XN
      PRINT 1001,NAME(NPA),XNA,NAME(NPB),XNB,SN,CFAC
 1001 FORMAT(,/,X,A10,* HAS *,F5.0,* AMINO ACIDS* ,/,X,A10,* HAS *
     +* AMINO ACIDS*,/,X,* SN = *,F7.1,/,X,*CORRECTION TERM = *,F7
     +,)
      PRINT 1002,SNN
 1002 FORMAT(* SN/N = *,F8.4,/,/,/,/,)
      PRINT 1003
 1003 FORMAT(,/,* DO YOU WANT TO KEEP THIS RESULT↓ *)
      READ 1004,ANS
 1004 FORMAT(A1)
      IF(ANS.EQ.≠Y≠)51,30
 51   WRITE(8,1001) NAME(NPA),XNA,NAME(NPB),XNB,SN,CFAC
      WRITE(8,1002) SNN
      GO TO 30
 99   STOP
      END
```

SAMPLE OUTPUT

```
SLPCC72     HAS   654. AMINO ACIDS
SLPCC58     HAS   526. AMINO ACIDS
 SN =    464.0
CORRECTION TERM =   -505.0

SN/N =     .7095



TC2.5SH     HAS   552. AMINO ACIDS
TC2.5SE     HAS   543. AMINO ACIDS
 SN =    230.5
CORRECTION TERM =      2.0

SN/N =     .4175



PCITA       HAS   552. AMINO ACIDS
PCITB       HAS   456. AMINO ACIDS
 SN =    699.8
CORRECTION TERM =   -271.2

SN/N =    1.2678



SKPC        HAS   993. AMINO ACIDS
SLPC        HAS   964. AMINO ACIDS
 SN =    158.5
CORRECTION TERM =      2.0

SN/N =     .1606
```

```
      PROGRAM SORT(INPUT,TAPE1=INPUT,OUTPUT,TAPE2=OUTPUT,TAPE60,
     + SORTED,TAPE6=SORTED,TAPE5=/30)
      DIMENSION P(17,20),X(20),F(17,25),NAME(30)
C
C
C     THIS PROGRAM READS THE PERCENTILE TABLE FOR A REFERENCE
C     SET OF PROTEINS FROM TAPE60.  IT THEN READS THE AMINO ACID
C     COMPOSITIONS AS MOLE% FOR EACH PROTEIN TO BE COMPARED
C     FROM TAPE5.  THE APPROPRIATE PERCENTILE VALUES FOR EACH
C     AMINO ACID IN EACH OF THESE PROTEINS IS OUTPUT TO A FILE
C     CALLED ≠SORTED≠.
C
C
      REWIND 5
      REWIND 60
   11 DO 200 I=1,17
      DO 200 J=1,20
  200 READ(60,*) P(I,J)
      NN=0
  100 CONTINUE
      NN=NN+1
      READ(5,*) NAME(NN),(X(I),I=1,17)
      IF (EOF(5)) 600,12
   12 DO 400 I=1,17
      IF(X(I).GT.P(I,20))40,50
   50 N=A=0
      DO 500 J=1,20
      B=P(I,J)
      IF(X(I).GT.B)30,20
   30 N=N+5
  500 A=B
   20 F(I,NN)=5*(X(I)-A)/(B-A)+N
      GO TO 400
   40 F(I,NN)=100.
  400 CONTINUE
      GO TO 100
  600 NN=NN-1
      DO 700 K=1,NN
      WRITE(2,1002) NAME(K),(F(I,K),I=1,17)
  700 WRITE(6,1003) NAME(K),(F(I,K),I=1,17)
 1003 FORMAT(A10,17F7.2)
 1002 FORMAT(1X,A10,17F7.2)
      STOP
      END
```

```
      PROGRAM CORCOEF(INPUT,TAPE1=INPUT,OUTPUT,TAPE2=OUTPUT,
     + SORTED,TAPE6=SORTED,TAPE8=/136)
      DIMENSION PTILE(17,30),R(30,30),NAME(30)
      COMMON X(17),Y(17)
C
CC
C
C
C    THIS PROGRAM READS AMINO ACID COMPOSITIONS AS PERCENTILES FROM
C    A FILE CALLED SORTED.  EACH PROTEIN IN THE SET IS COMPARED TO
C    EACH OTHER PROTEIN IN THE SET AND A CORRELATION COEFFICIENT IS
C    CALCULATED.  THE CORRELATION COEFFICIENTS ARE OUTPUT AS A TABLE
C    TO A FILE CALLED TAPE8.
C
C
C
      REWIND 6
      PRINT 1000
 1000 FORMAT(,/,* HOW MANY PROTEINS IN THE SET↓ *,/)
      READ*, N
      DO 100 J=1,N
  100 READ(6,1020) NAME(J),(PTILE(I,J),I=1,17)
 1020 FORMAT(A10,17F7.2)
      NN=N-1
      DO 300 J=1,NN
      DO 200 I=1,17
  200 X(I)=PTILE(I,J)
      JJ=J+1
      DO 300 K=JJ,N
      DO 400 I=1,17
  400 Y(I)=PTILE(I,K)
      CALL COR (CC)
  300 R(J,K)=CC
      DO 500 J=1,NN
      WRITE(2,1005) NAME(J), (R(J,K),K=2,N)
 1005 FORMAT(1X,A10,21F6.4)
      WRITE(8,1005) NAME(J),(R(J,K), K=2,N)
  500 WRITE(8,99)
   99 FORMAT(,/)
      STOP
      END
      SUBROUTINE COR(CC)
      COMMON X(17),Y(17)
      SXY=SX=SY=SX2=SY2=0
      DO 100 I=1,17
      SX=SX+X(I)
  100 SY=SY+Y(I)
      SX=SX/17.
      SY=SY/17.
      DO 10 I=1,17
      XY=(SX-X(I))*(SY-Y(I))
      SXY=SXY+XY
      Y2=(SY-Y(I))**2
      SY2=SY2+Y2
      X2=(SX-X(I))**2
   10 SX2=SX2+X2
      CC=SXY/SQRT(SX2*SY2)
      RETURN
      END
```

```
      PROGRAM PHYLO(INPUT,OUTPUT,TAPE6=/80,TAPE5=/80,TAPE8)
      COMMON/ONE/TABL(25,17),P(17,20),F(25,17)
      COMMON/FOUR/X(17),Y(17)
      DIMENSION R(24,24),NAME(25),T(25)
C
C
C
C     THIS PROGRAM READS THE PERCENTILE TABLE FOR A REFERENCE
C     SET OF PROTEINS FROM TAPE6 AND THE AMINO ACID COMPOSITIONS
C     AS MOLE% FOR EACH PROTEIN TO BE COMPARED FROM TAPE5.
C     OUTPUT IS TO TAPE8.
C
C
C
      REWIND 5
      REWIND 6
      DO 101 I=1,17
      DO 101 J=1,20
  101 READ(6,*) P(I,J)
      NPR=1
  203 READ(5,*) NAME(NPR),(TABL(NPR,I),I=1,17)
      IF(EOF(5)) 201,202
  202 T(NPR)=1
      NPR=NPR+1
      GOTO 203
  201 NPR=NPR-1
      NN=NPR-1
      DO 204 J=1,NPR
  204 CALL SORT(J)
  207 AMAXR=0
      DO 300 J=1,NN
      X(1)=F(J,1)
      IF(X(1).EQ.0)215,701
  701 DO 200 I=2,17
  200 X(I)=F(J,I)
      JJ=J+1
      DO 301 K=JJ,NPR
      Y(1)=F(K,1)
      IF(Y(1).EQ.0)216,704
  704 DO 400 I=2,17
  400 Y(I)=F(K,I)
      CALL COR (CC)
      R(J,K)=CC
      AMAXR=AMAX1(AMAXR,R(J,K))
      GO TO 301
  216 R(J,K)=ZILCH
  301 CONTINUE
      GO TO 300
  215 DO 206 M=1,NPR
  206 R(J,M)=ZILCH
  300 CONTINUE
      PRINT 1011
 1011 FORMAT(*              2   3   4   5   6   7   8   9   10 11 12 13 14 15 16
     +* 17 18 19 20 21*)
      WRITE(8,1009)
 1009 FORMAT(,/,18X,* 2      3      4      5      6      7      8      9*
     +*      10     11     12     13     14     15     16     17     18*
     +*     19     20     21 *)
      DO 500 J=1,NN
      PRINT1005,J,NAME(J),(R(J,K),K=2,NPR)
 1005 FORMAT(1X,I3,A6,24F3.2)
  500 WRITE(8,1006) J,NAME(J),(R(J,K),K=2,NPR)
      WRITE(8,1006) NPR,NAME(NPR)
 1006 FORMAT(1X,I3,1X,A10,24F6.4,/)
      PRINT 1007,AMAXR
 1007 FORMAT(1X,* HIGHEST CORRELATION COEFFICIENT = *,F5.3)
      WRITE(8,1007) AMAXR
      PRINT 1010
 1010 FORMAT(1X,* WHICH PROTEINS ARE TO BE GROUPED+ *,/,)
      READ*,NAME1,NAME2
      IF(NAME1.EQ.0) 99,205
  205 DO 102 I=1,17
  102 TABL(NAME1,I)=(TABL(NAME1,I)*T(NAME1)+TABL(NAME2,I)*T(NAME2))
     +  /(T(NAME1)+T(NAME2))
      T(NAME1)=T(NAME1)+T(NAME2)
      F(NAME2,1)=0
      WRITE(8,1008) NAME1,NAME2
 1008 FORMAT(,/,/,/,/,/,/,/,1X,*PROTEINS*,I3,* AND*,I3,* HAVE BEEN GROUP
     +.*)
      CALL SORT(NAME1)
      GO TO 207
   99 STOP
      END
C
C
```

```
C
C
      SUBROUTINE SORT(J)
C
C        THIS SUBROUTINE CONVERTS THE MOLE% VALUES IN ARRAY
C        *TABL* TO PERCENTILES, WHICH ARE STORED IN ARRAY *F*.
C
C
C
      COMMON/ONE/TABL(25,17),P(17,20),F(25,17)
      DO 400 I=1,17
      IF(TABL(J,I).GT.P(I,20))40,50
 50   N=A=0
      DO 500 K=1,20
      B=P(I,K)
      IF(TABL(J,I).GT.B)30,20
 30   N=N+5
 500  A=B
 20   F(J,I)=5*(TABL(J,I)-A)/(B-A)+N
      GO TO 400
 40   F(J,I)=100.
 400  CONTINUE
      RETURN
      END
C
C
C
C
      SUBROUTINE COR(CC)
      COMMON/FOUR/X(17),Y(17)
      SX=SY=SXY=SX2=SY2=0
      DO 100 I=1,17
      SX=SX+X(I)
 100  SY=SY+Y(I)
      SX=SX/17.
      SY=SY/17.
      DO 10 I=1,17
      XY=(SX-X(I))*(SY-Y(I))
      SXY=SXY+XY
      Y2=(SY-Y(I))**2
      SY2=SY2+Y2
      X2=(SX-X(I))**2
 10   SX2=SX2+X2
      CC=SXY/SQRT(SX2*SY2)
      RETURN
      END
```

CHAPTER 6

THE TRANSLOCATION OF CARBOXYBIOTIN IN PYRUVATE CARBOXYLASE

6.1 INTRODUCTION

The current view of the active site of pyruvate carboxylase is that it consists of two spatially distinct sub-sites. At the first sub-site, the fixation of CO_2 to form an N-carboxybiotin complex is coupled to the hydrolysis of ATP:

$$\text{ENZ-biotin} + MgATP^{2-} + HCO_3^- \rightleftharpoons \text{ENZ-biotin-}CO_2^- + MgADP^- + Pi^{--} \quad ..(i)$$

Once formed, the N-carboxybiotin intermediate acts as a mobile carboxyl-group carrier and transports the activated carboxyl group to the second sub-site where pyruvate binds and the second partial reaction occurs:

$$\text{ENZ-biotin-}CO_2^- + \text{pyruvate} \rightleftharpoons \text{ENZ-biotin} + \text{oxaloacetate} \quad ..(ii)$$

Initial velocity and product inhibition studies have been used to elucidate the order of substrate binding and product release (McClure *et al.*, 1971b; Barden *et al.*, 1972; Warren & Tipton, 1974; Easterbrook-Smith *et al.*, 1978) but these investigations provide very little information about the nature of the interactions and intermediate steps that give rise to product formation. Isotope-exchange procedures have been used to provide some insight into the sequence of events occurring at each of the two sub-sites (McClure *et al.*, 1971c; Scrutton & Utter, 1965; Ashman & Keech, 1975). However, the only information available on the translocation of the carboxybiotin from the first sub-

site to the second sub-site comes from the study of Easterbrook-Smith *et al.* (1976a). They showed that pyruvate carboxylase exhibits a hydrolytic leak, i.e., an abortive hydrolysis of the ENZ-carboxybiotin complex at low concentrations of pyruvate. The explanation for this phenomenon put forward by Easterbrook-Smith *et al.* is that the binding of pyruvate shifts the carboxybiotin to the pyruvate binding site in readiness for the carboxylation of pyruvate. Previously, the NMR studies of Mildvan *et al.* (1966) had shown that the binding of pyruvate is a rapid equilibrium process and that pyruvate moves into and out of the pyruvate binding site at a rate which is two orders of magnitude faster than the rate of the overall reaction. Therefore, if the carboxybiotin arrives at the pyruvate binding site and finds the site unoccupied - a likely event at non-saturating levels of pyruvate - some of the complex may spontaneously hydrolyse to biotin and CO_2. The study on the isotope effects on pyruvate carboxylase catalysis by Cheung & Walsh (1976) supports this explanation. They concluded that "after the pyruvate molecule binds, it is only about 50% committed to catalysis, i.e., it will come back off the enzyme without reacting one out of two times it binds". The important points are, firstly, that it is the binding of pyruvate that acts as a signal to summon carboxybiotin into the second sub-site, and secondly, that carboxybiotin is very labile once it is bound at the second sub-site. The experiments described in this chaper examine the factors that influence the location of carboxybiotin.

6.2 METHODS

6.2.1 Assessing 2-oxobutyrate purity

The purity of 2-oxobutyrate was checked by thin layer chromatography of its dinitrophenylhydrazone on a cellulose thin layer plate, using butan-1-ol/water/ethanol (5:4:1 by vol) as the developing solvent. This system separates the dinitrophenylhydrazones of pyruvate and 2-oxobutyrate. No contaminants were detected.

6.2.2 Isolation of the ENZ-[^{14}C]-carboxybiotin complex

Pyruvate carboxylase was incubated in 0.5 ml of a solution containing 100 mM N-ethylmorpholine-HCl, pH 7.8, 5 mM $MgCl_2$, 0.25 mM acetyl CoA, 2.5 mM ATP and 2.5 mM $NaH^{14}CO_3$ (57 mCi/mmole). After 10 min at 0°C, the reaction was terminated by the addition of 0.5 ml of 200 mM EDTA, pH 7.0, After standing for at least 20 min at 0°C, the ENZ-[^{14}C]-carboxybiotin complex was transferred to a 100 mM N-ethylmorpholine-HCl solution, pH 7.8, by centrifuging through Sephadex G-25 as described by Helmerhorst & Stokes (1980).

6.2.3 Measurement of the rate of hydrolysis of ENZ-[^{14}C]-carboxybiotin

Samples of the isolated ENZ-[^{14}C]-carboxybiotin were incubated under the conditions described in the text and at various time intervals, a 20 µl portion was transferred to a tube containing 20 µl of 20 mM pyruvate in 0.1 M N-ethylmorpholine-HCl, pH 7.8, for 5 min at 4°C. The product, oxaloacetate, was stabilized with 20 µl of 2 M semicarbazide hydrochloride and 50 µl of the final solution was applied to a 2 cm x 2 cm square of Whatman

3 MM paper. The paper was heated at 100°C for 5 min to remove any $^{14}CO_2$ not incorporated in oxaloacetate and counted in 3 ml of toluene scintillation fluid.

6.2.4 Measurement of the translocation rate

Samples (200 µl containing 100,000 cpm/ml) of the isolated ENZ-[^{14}C]-carboxybiotin were incubated for 10 min at 0°C in the presence of the compound to be tested. The measurement of the translocation rate was initiated by the addition of 20 mM 2-oxobutyrate. At various time intervals, 20 µl samples were transferred to a tube containing 40 µl of 2 M semicarbazide hydrochloride. A 50 µl portion of this solution was applied to a 2 cm x 2 cm square of Whatman 3 MM paper, which was heated at 105°C for 5 min to remove any $^{14}CO_2$ not transferred to 2-oxobutyrate and the radioactivity of the paper was counted in 3 ml of toluene scintillation fluid.

At the beginning and end of each experiment, the total concentration of the ENZ-[^{14}C]-carboxybiotin complex was measured by transfer of the [^{14}C]-carboxyl group to pyruvate.

6.2.5 Data analysis

Lines were fitted by the weighted least-squares method with the aid of the GLIM system (Baker & Nelder, 1978) to determine the simplest model structure and to obtain maximum likelihood estimates of parameters.

Throughout the analysis, the fitting of lines was done by converting all relations to a linear form and performing a least squares linear regression using appropriate weights (i.e., the weight of $f(x)$ is $[f'(x)]^{-2}(\mathrm{Var}(x))^{-1}$].

6.2.5.1 Fitting the primary data: There were two operations involved in measuring the rate of transfer of $^{14}CO_2$ from ENZ-[^{14}C]-carboxybiotin to 2-oxobutyrate. These were (a) determining the total initial ENZ-[^{14}C]-carboxybiotin by transfer to pyruvate (let this = C_o) and (b) determining the amount of $^{14}CO_2$ which had been transferred to 2-oxobutyrate at time "t" (let this = X_t). From C_o and X_t we obtain the proportion of ENZ-[^{14}C]-carboxybiotin remaining at time "t" (let this = C_t)

$$C_t = \frac{C_o - X_t}{C_o} = 1 - \frac{X_t}{C_o}$$

There are two sources of error in X_t - experimental error (such as volume errors) and radioactive counting errors. Experimental error does not vary with t, as each sample is handled in the same way, and so this need not be included in the weights allotted to each C_t. As counting errors vary with the size of X_t, they should be taken into account in the weighting of each C_t. As radioactive decay follows a Poisson distribution, the variance of X_t due to counting error will be X_t.

As the transfer process was expected to be pseudo-first order, ln (C_t) was plotted against t with weights of $\frac{C_o}{X_t}\left(1 - \frac{X_t}{C_o}\right)^2$

6.2.5.2 Secondary plots: Each plot of ln (C_t) versus t generates two secondary parameters - the intercept on the ordinate and the slope. The interpretation of these is

discussed in Section 6.3.1, where the slope is identified with the rate constant of translocation of carboxybiotin to the second subsite and the initial proportion of unbound carboxybiotin is called the fractional burst, obtained from $1 - e^i$ where i is the intercept on the ordinate.

Equations xii and viii of Section 6.3.4 described relations between the Mg^{2+} concentration and the rate and fractional burst respectively.

$$\frac{1}{\text{rate (constant)}} = \frac{k_{-1}}{k_1 k_2'} \left[Mg^{2+}\right] + \frac{1}{k_2'} \quad ..(xii)$$

$$\frac{1}{\text{fractional burst}} = \frac{k_1 k_2}{k_{-1} k_{-2}} \left[Mg^{2+}\right] + \frac{k_{-2}}{k_2} + 1 \quad ..(viii)$$

In plotting 1/rate against $[Mg^{2+}]$ (Fig. 6.6), weights were assigned as follows. The variance of the rate [Var(r)] is the variance of the slope of the primary plot, and

$$\text{Var}\left(\frac{1}{r}\right) = \frac{1}{r^2} \text{Var}(r)$$

so weight (r) = $r^2/\text{Var}(r)$

where r represents the rate.

In the burst replot (Fig. 6.5), the weights were calculated as follows:

fractional burst = $1 - e^i$

1/fractional burst = $1/(1 - e^i)$

$$\text{Var (1/fractional burst)} = \left[\frac{e^i}{(1 - e^i)^2}\right]^2 \text{Var}(i)$$

In both the 1/rate and 1/fractional burst secondary plots the fitting was refined by recalculating the weights using the fitted values of 1/rate or 1/fractional burst and repeating the fitting procedure in an iterative manner until there was no significant change in the fitted line. As all the points had been initially weighted with the reciprocal of their measured variances, the overall variance of each regression was distributed as χ^2 (Bliss, 1970) and so goodness of fit could be tested using a χ^2 test with the appropriate degrees of freedom.

6.2.5.3 Testing the effect of acetyl-CoA: The GLIM system will fit any model of linear structure to data and calculate the deviance, which for the normal distribution is the residual sum of squares. Analysis of variance and χ^2 tests were used to test whether each component of the model structure provided a significant improvement in the goodness of fit. The simplest linear model structure which adequately fitted the data was thereby obtained.

6.3 RESULTS AND DISCUSSION

6.3.1 Time course for the carboxylation of 2-oxobutyrate from ENZ-[^{14}C]-carboxybiotin

Since the transfer of the activated carboxyl group from the ENZ-carboxybiotin form to pyruvate is very rapid, an analogue of pyruvate, 2-oxobutyrate, and a low temperature (0°C) were used to slow down the transfer process. By using this assay system it was possible to study the factors controlling the release of the N-carboxybiotin complex from the first sub-site. The carboxylation product

of 2-oxobutyrate has been identified by Kerr (1965) as β-methyloxaloacetate.

Figure 6.1 presents the data obtained when the isolated ENZ-[^{14}C]-carboxybiotin complex was treated with 10 mM EDTA and the time course for the transfer of the activated carboxyl group to 2-oxobutyrate was determined. Although the reaction appears to obey pseudo-first-order kinetics, the line of best fit does not extrapolate to 100% when t = 0. This result has been interpreted to indicate that there are two forms or states of the ENZ-[^{14}C]-carboxybiotin complex at equilibrium with each other; one state is proposed to be enzyme with carboxybiotin bound at the first sub-site (State I) and the other state (State II) is unbound, presumably in the vicinity of the first sub-site (certainly not in the second sub-site where the carboxybiotin is known to be unstable; Easterbrook-Smith *et al.*, 1976a).

$$\underset{\text{(bound)}}{\text{State I}} \rightleftharpoons \underset{\text{(unbound)}}{\text{State II}} \qquad \ldots\text{(iii)}$$

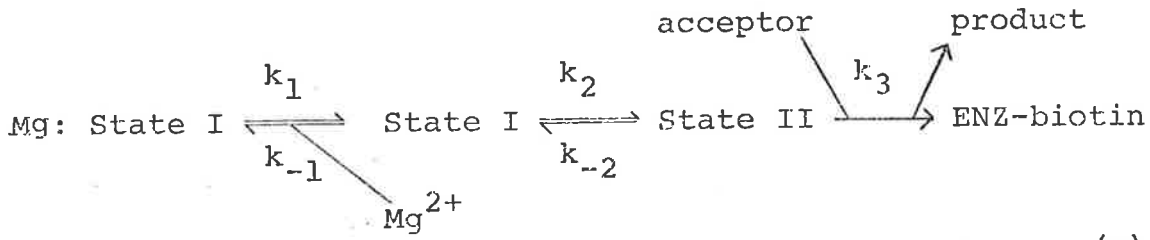
After the initial burst of transferring activity (Fig. 6.1), presumably due to the transfer of the activated carboxyl group from State II to the acceptor molecule (equation iv), a second, lower, rate of transfer is observed.

$$\text{State I} \rightleftharpoons \text{State II} \xrightarrow[\text{acceptor}]{} \text{ENZ-biotin} \qquad \ldots\text{(iv)}$$
(product)

Since the transfer from State II to acceptor is very rapid, the second slower phase has been interpreted to represent the rate of dissociation of carboxybiotin bound at the first sub-site (State I) to form State II.

6.3.2 Effect of Mg^{2+} on the ENZ-[^{14}C]-carboxybiotin complex

Any treatment that perturbs the equilibrium between the two states will affect both the size of the "burst" and the observed rate of the transfer process. Results presented in Fig. 6.2 show that Mg^{2+} has a pronounced effect on both processes. The decrease in the "burst" size with increasing concentrations of Mg^{2+} indicates that the equilibrium between the two states has been shifted in favour of State I, whereas the decrease in the observed transfer rate indicates that in the presence of Mg^{2+}, State I carboxybiotin is bound more tightly to the first sub-site (equation v).



...(v)

6.3.3 Model equations

In order to develop this model in quantitative terms, the following assumptions were made:

(a) in the absence of a carboxyl group acceptor molecule (eqn. v), the [^{14}C]-carboxybiotin moiety adopts an equilibrium distribution between State I and State II, i.e.,

$$k_2[\text{State I}] = k_{-2}[\text{State II}] \qquad \ldots(vi)$$

and

$$k_{-1}[Mg^{2+}][\text{State I}] = k_1[\text{Mg:State I}] \qquad \ldots(vii)$$

(b) the interconversion of State I and State II is slow, i.e., k_{-2}, $k_2 \ll k_1$, k_{-1} and k_3, and

(c) 2-oxobutyrate is present at saturating concentrations.

Thus, the fraction of total [^{14}C]-carboxybiotin converted into stable products in the rapid-burst phase of the reaction, i.e., the fractional burst, equals

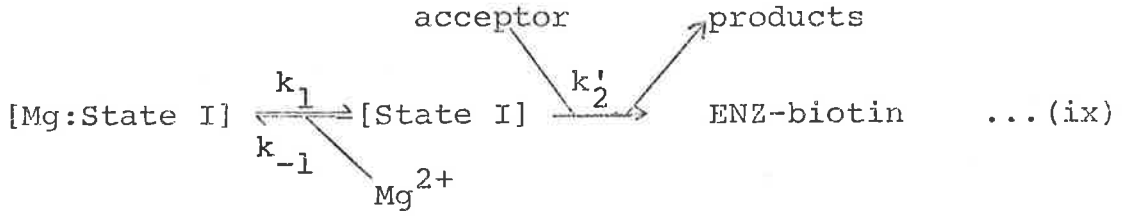
$$\frac{[\text{State II}]}{[\text{Mg:State I}] + [\text{State I}] + [\text{State II}]}$$

$$= \frac{[\text{State II}]}{\frac{k_{-1}\, k_{-2}}{k_1\, k_2}[\text{Mg}][\text{State II}] + \frac{k_{-2}}{k_2}[\text{State II}] + [\text{State II}]}$$

and

$$\frac{1}{\text{Fractional burst}} = \frac{k_{-1}k_{-2}}{k_{+1}\, k_{+2}}[Mg^{2+}] + \frac{k_{-2}}{k_{+2}} + 1 \qquad \ldots(viii)$$

After the rapid phase of the reaction, virtually all remaining [^{14}C]-carboxybiotin is bound at the first sub-site, i.e. [State II] $\longrightarrow$ 0. Furthermore, from the assumptions (b) and (c), k_2 is the rate-limiting step during the conversion of [^{14}C]-carboxybiotin into stable products during the slow phase so that eqn. (v) simplifies to:



...(ix)

with k_2' rate limiting.

From assumption (b), at time "t",

$$k_1[\text{Mg:State I}] = k_{-1}[Mg^{2+}][\text{State I}]$$

and total [^{14}C]-carboxybiotin at time "t"

$$= [\text{Mg:State I}] + [\text{State I}]$$

$$= k_{-1}/k_1 [Mg^{2+}][\text{State I}] + [\text{State I}]$$

$$= [\text{State I}] \left[\frac{k_{-1}}{k_1} [Mg^{2+}] + 1 \right]$$

Thus, $$\frac{d([^{14}C]\text{-carboxybiotin})}{dt} = k_2'[\text{State I}]$$

$$= -\frac{k_2'[^{14}C]\text{-carboxybiotin}}{1 + \frac{k_{-1}}{k_1}[Mg^{2+}]} \qquad \ldots(x)$$

Integration gives:

$$\ln \left[\frac{[^{14}C]\text{-carboxybiotin}_t}{[^{14}C]\text{-carboxybiotin}_o} \right] = - \frac{k_2'}{1 + \frac{k_{-1}}{k_1}[Mg^{2+}]} t \qquad \ldots(xi)$$

Thus the apparent first order rate constant is:

$$\frac{k_2'}{1 + \frac{k_{-1}}{k_1}[Mg^{2+}]}$$

so that 1/rate = (constant) $$\frac{k_{-1}}{k_1 k_2'}[Mg^{2+}] + \frac{1}{k_2'} \qquad \ldots(xii)$$

Thus, a plot of 1/rate against $[Mg^{2+}]$ should be linear with slope of $k_{-1}/k_1 k_2'$ and intercept of $1/k_2'$, whereas a plot of

1/fractional burst against $[Mg^{2+}]$ should also be linear but with a slope of $k_{-1}\ k_{-2}/k_1\ k_2$ and an intercept of $1 + k_{-2}/k_2$. Graphs of this form are shown in Figs. 6.3 and 6.4 and fit the prediction of linearity ($P > 10\%$, χ^2 test; Bliss, 1970). From a fractional burst plot it can be calculated that in the absence of Mg^{2+} the equilibrium between the two states, i.e., k_2/k_{-2}, is 0.85 ± 0.08.

6.3.4 Effect of acetyl-CoA on the ENZ-[^{14}C]-carboxybiotin complex

In order to ascertain whether the allosteric activator of pyruvate carboxylase, acetyl-CoA, influenced events involved in the translocation process, an experiment similar to that shown in Fig. 6.2 was performed in the presence of 250 μM acetyl-CoA (Fig. 6.5). Table 6.1 shows the variances associated with four models of possible affects of acetyl-CoA on the secondary plots of 1/fractional burst (Fig. 6.3) and 1/rate (Fig. 6.4) against $[Mg^{2+}]$, which are based on equations (viii) and (xii). Also shown in Table 6.1 are the χ^2 (goodness of fit) tests on these models. Table 6.2 shows the results of analysis of variance (F tests) on the models. It is evident from both tests that, in the 1/rate plot, acetyl-CoA affects the slope but not the intercept on the ordinate. In the fractional burst plot none of the models could be rejected on the basis of the χ^2 test, but the F test shows that independent lines fit the data significantly better than parallel lines, whereas independent lines do not fit significantly better than lines intersecting on the ordinate. Thus in the simplest model which

fits the data acetyl-CoA affects the slope, but not the intercept on the ordinate.

In both secondary plots (Figs. 6.3 and 6.4) the presence of acetyl-CoA affects the slope of the plot but not the intercept on the ordinate, thus indicating that the dissociation constant for Mg^{2+} (k_1/k_{-1}) is increased. The effect of this is to increase the concentration of the State II form, which provides more N-carboxybiotin for transfer to the acceptor substrate. On the other hand, acetyl-CoA has no significant effect on k_2/k_{-2}, i.e., on the equilibrium between State I and State II. The estimated values of k_1/k_{-1}, k_2/k_{-2} and k_2' are shown in Table 6.3. Bais & Keech (1972) have found the dissociation constant for free Mg^{2+} to be dependent on the temperature. Extrapolation of their Arrhenius plot to 0°C yeilds a dissociation constant of 2.5 mM at this temperature. This is in reasonable agreement with the value of 5.6 ± 1.0 mM obtained in the present study.

6.3.5 Testing alternative interpretations

When the carboxylation of 2-oxobutyrate from ENZ-carboxybiotin is monitored at concentrations of 2-oxobutyrate above 20 mM, there is only a small change in the time course. These changes are minor when compared to the profound effect of Mg^{2+} on the time courses (Fig. 6.6). Thus we can conclude firstly that 2-oxobutyrate is nearly saturating at 20 mM and secondly that the burst phenomenon is not due to contamination of the 2-oxobutyrate with pyruvate. The purity of the 2-oxobutyrate was also assessed by thin layer chromatography, as described in the Methods

section. No contaminants could be detected.

The effects of increasing Mg^{2+} concentration seen in Fig. 6.2 allow us to eliminate several other possible explanations for the existence of a burst in the carboxyl-transfer process. The slower rate which is observed cannot be due to residual ATP and $H^{14}CO_3^-$ as there is an absolute requirement for Mg^{2+} for carboxylation of the enzyme from ATP and HCO_3^- (Bais & Keech, 1972), yet the maximal rate of the transfer reaction is observed in the presence of 10 mM EDTA. The fact that the magnitude of the burst varies with Mg^{2+} concentration (with Mg^{2+} added only a few minutes before initiation of the transfer reaction), also means that the burst is not due to some permanent heterogeneity in the enzyme population.

The effects of increasing Mg^{2+} concentration observed in Fig. 6.2 cannot be attributed to changes in Cl^- concentration or ionic strength because the time course for the transfer to 2-oxobutyrate in the presence of 18 mM NaCl is similar to that obtained in the presence of 10 mM EDTA (Fig. 6.7). Furthermore, the half-life of the ENZ-[^{14}C]-carboxybiotin complex prepared using the same conditions as the experiments described above and stored in the absence of a CO_2^--acceptor molecule (i.e., in the absence of either pyruvate or 2-oxobutyrate) was found to depend on the Mg^{2+} concentration (Fig. 6.8). In the absence of Mg^{2+} the half-life was 50 min whereas in the presence of 16 mM Mg^{2+} the half-life was 105 min. The 2-fold increase in the half-life time of the complex indicates that the carboxybiotin moiety is more stable in the first sub-site and is probably com-

plexed to the Mg^{2+}.

As Mg^{2+} holds carboxybiotin in the first sub-site, one would expect high concentrations of Mg^{2+} to inhibit the overall activity of the enzyme. Figure 6.9 shows that Mg^{2+} is inhibitory at concentrations above 1.6 mM.

6.3.6 Transcarboxylation by chicken liver pyruvate carboxylase

Chicken liver pyruvate carboxylase undergoes a loss of activity on exposure to low temperatures (2°C) (Scrutton *et al.*, 1965). Thus it is not possible to thoroughly study the translocation of carboxybiotin in the chicken enzyme using the methods employed with the sheep enzyme. However, the chicken enzyme is protected from cold inactivation by 70 μM acetyl-CoA (Irias *et al.*, 1969) and so it was possible to verify that in the presence of acetyl-CoA, Mg^{2+} has the same qualitative effects on the translocation of carboxybiotin in chicken liver pyruvate carboxylase as on the enzyme from sheep liver (Fig. 6.10).

TABLE 6.1

THE VARIANCES ASSOCIATED WITH DIFFERENT MODELS OF THE AFFECT OF ACETYL-CoA

MODEL	DEGREES OF FREEDOM	VARIANCE	$P(\chi^2)$
FRACTIONAL BURST PLOT			
Single line (no effect)	10	14.22	>10%
lines intersecting on the ordinate (affects slope)	9	11.44	>10%
parallel lines (affects intercept)	9	14.42	>10%
separate lines (affects slope and intercept)	8	8.27	>10%
RATE PLOT			
single line	10	42.86	<0.1%*
lines intersecting on ordinate	9	11.93	>10%
parallel lines	9	23.97	<0.5%*
separate lines	8	13.04	>10%

* The model can be rejected on χ^2 test.

TABLE 6.2

ANALYSIS OF VARIANCE ON MODELS OF THE EFFECT OF ACETYL-CoA

MODELS UNDER TEST	DEGREES OF FREEDOM (ν_1, ν_2)	$F\nu_1\nu_2$	$P(F\nu_1\nu_2)$	CONCLUSION
FRACTIONAL BURST PLOT				
single line versus lines intersecting on on ordinate	1, 9	2.18	>5%	
single line v parallel lines	1, 9	0	>5%	
intersecting v separate lines	1, 8	3.06	>5%	
parallel v separate lines	1, 8	5.94	2.5%<P< 5%	reject parallel lines
single line v separate lines	2, 8	2.87	>5%	
RATE PLOT				
single v intersecting lines	1, 9	23.32	<0.1%	reject single line
intersecting v separate lines	1, 8	0	>5%	
parallel v separate lines	1, 8	6.71	2.5%<P< 5%	reject parallel lines

TABLE 6.3

ESTIMATES OF k_1/k_{-1}, k_2/k_{-2} AND k'_2

SOURCE OF ESTIMATE	ACETYL-CoA	k_1/k_{-1} (mM)	k_2/k_{-2}	k'_2 (min^{-1})
Rate plot	-	2.8 ± 0.5		)
				) 0.073 ± 0.007
	+	5.3 ± 1.1		)
Burst plot	-	5.3 ± 1.4)		
		)	0.85 ± 0.08	
	+	6.4 ± 3.2)		
Rate and Burst	-	3.5 ± 0.6		
combined	+	5.6 ± 1.0		

The constants were obtained from plots of 1/fractional burst and 1/rate against $[Mg^{2+}]$, based on equations viii and xii respectively.

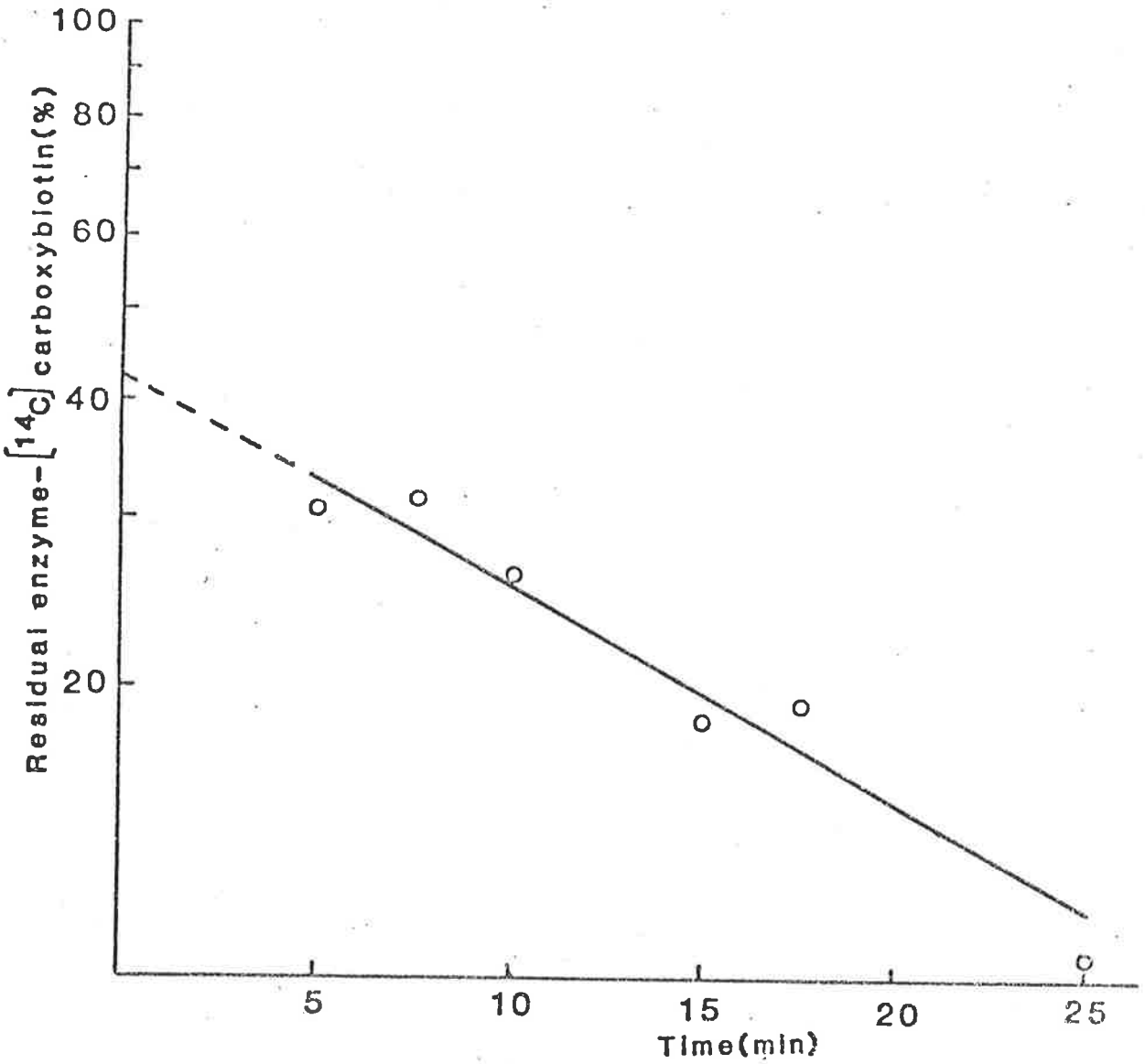


FIGURE 6.1

TIME COURSE OF THE TRANSLOCATION OF THE [^{14}C]-CARBOXY-BIOTIN COMPLEX AND THE TRANSFER OF THE ACTIVATED CARBOXY GROUP TO 2-OXOBUTYRATE BY PYRUVATE CARBOXYLASE

The assay solution contained: 100 mM N-ethylmorpholine/HCl buffer, pH 7.8, Enzyme-[^{14}C]-carboxybiotin complex (10^5 cpm/ml) and 10 mM EDTA at 0°C. The reaction was initiated by the addition of 20 mM 2-oxobutyrate. At specific time intervals, 20 µl samples were transferred to 40 µl of 2 M semicarbazide hydrochloride, and the amount of acid-stable radioactivity was determined. The total amount of radioactivity available at any time was determined by transferring any unreacted [^{14}C]-carboxybiotin to pyruvate, as described in the Methods and Materials section.

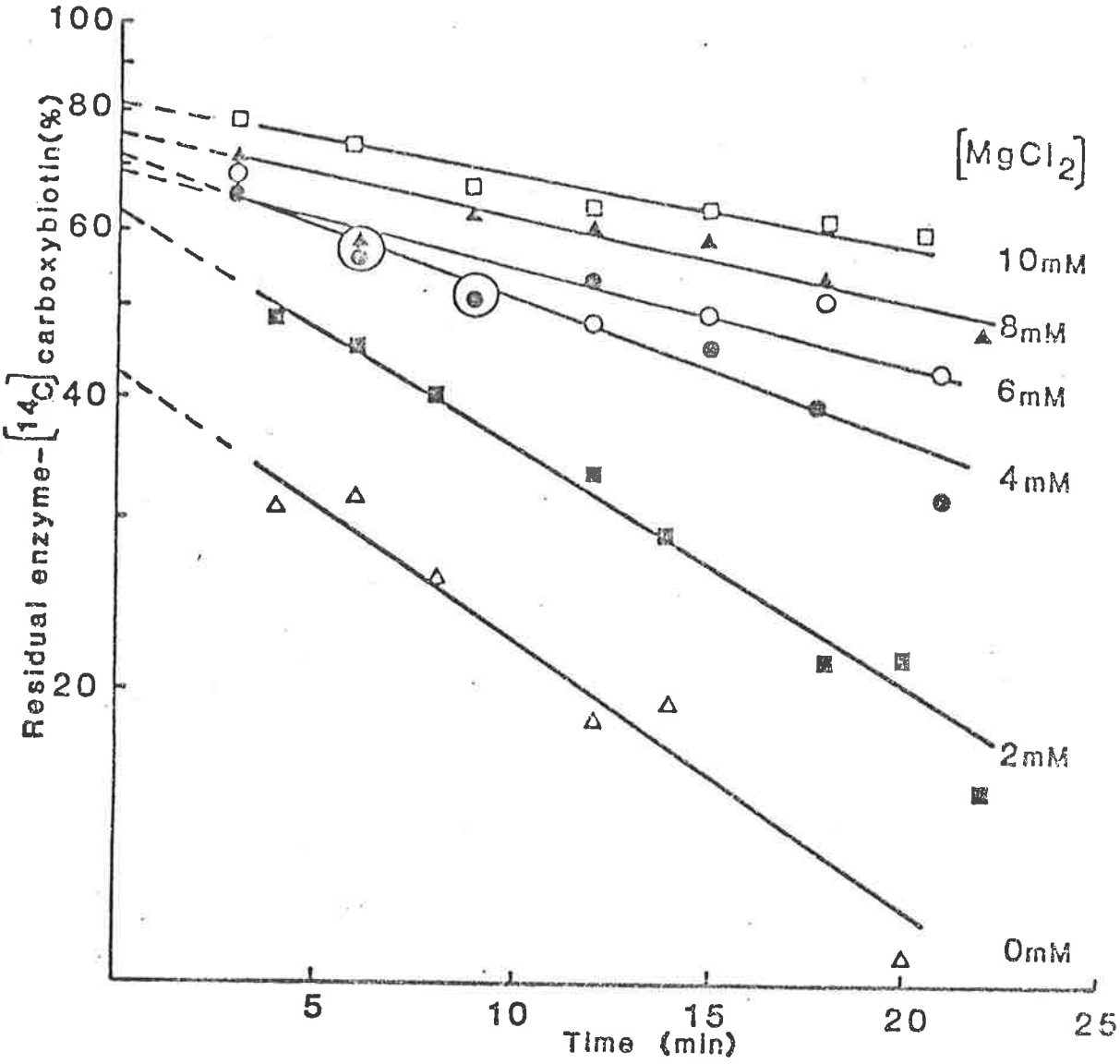


FIGURE 6.2

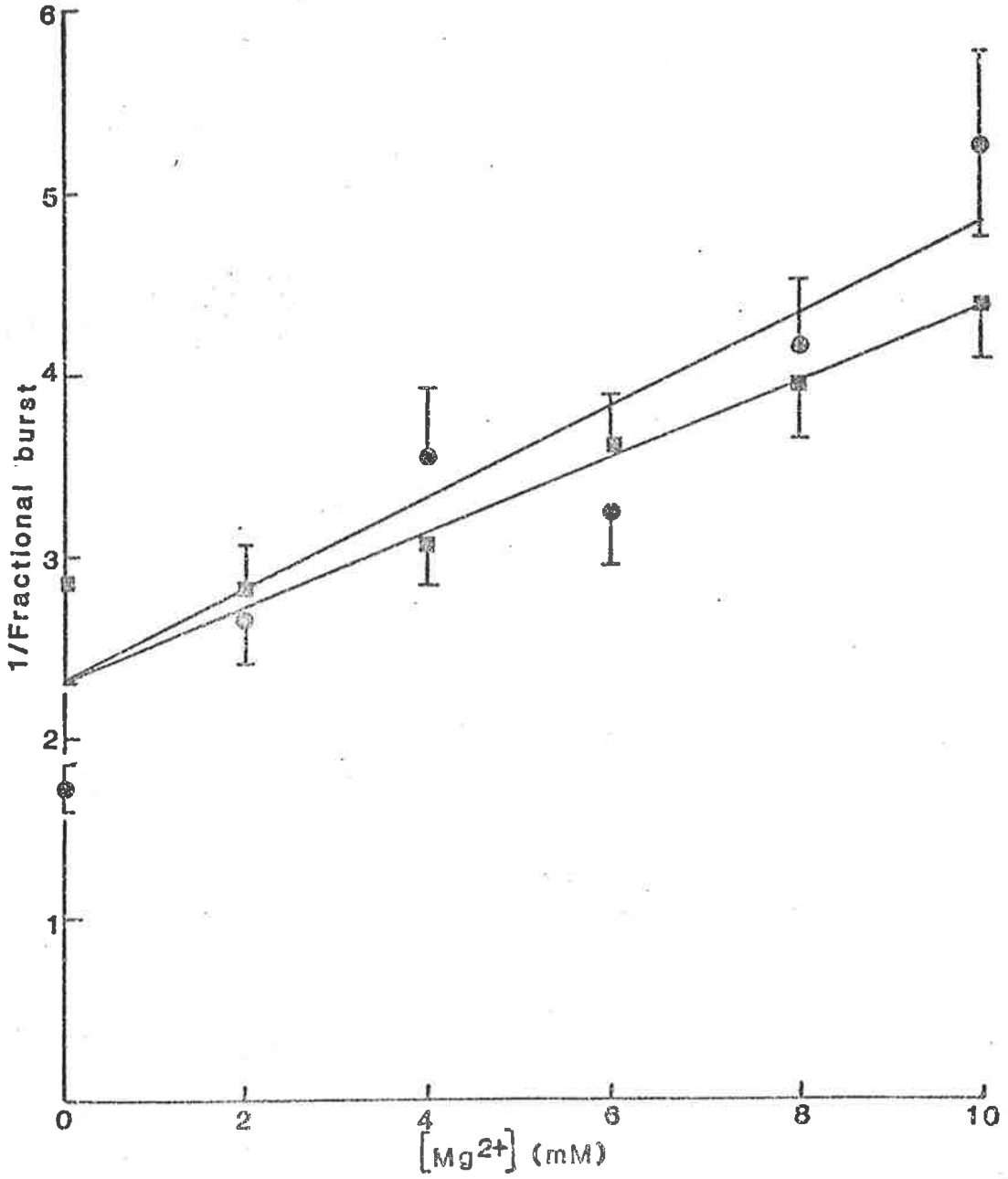
TIME COURSE OF THE TRANSLOCATION AND TRANSFER PROCESSES BY PYRUVATE CARBOXYLASE IN THE PRESENCE OF Mg^{2+}

The experimental conditions were the same as those described in Fig. 6.1 legend, except that Mg^{2+} was added as follows: 2 mM (■); 4 mM (●); 6 mM (○); 8 mM (▲): 10 mM (□). The control contained no Mg^{2+}, but 10 mM EDTA (△).

FIGURE 6.3

SECONDARY PLOT WHERE THE RECIPROCAL OF THE FRACTIONAL BURST OF PYRUVATE CARBOXYLASE IS PLOTTED AS A FUNCTION OF Mg^{2+} CONCENTRATION

The reciprocal of the ordinate intercepts were obtained from Fig. 6.2 (●) and Fig. 6.5 (■) and were plotted in accordance with eqn. (8). The error bars indicate ± 1 S.D.



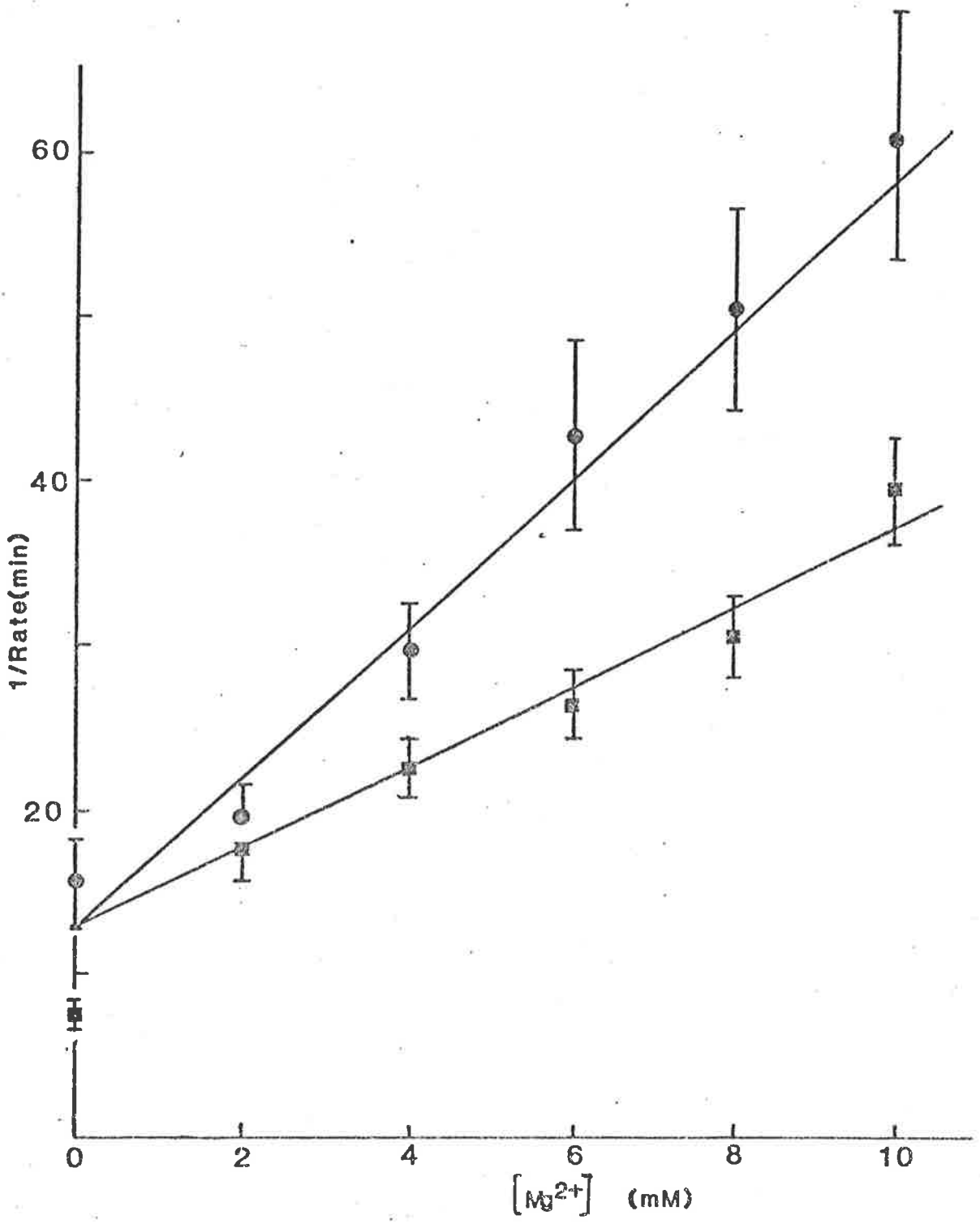


FIGURE 6.4

SECONDARY PLOT WHERE THE RECIPROCAL OF THE RATE OF TRANSLOCATION BY PYRUVATE CARBOXYLASE IS PLOTTED AS A FUNCTION OF Mg^{2+} CONCENTRATION

The reciprocals of the rates were obtained from Fig. 6.2 (●) and Fig. 6.5 (■) and were plotted in accordance with eqn (12). The error bars indicate ± 1 S.D.

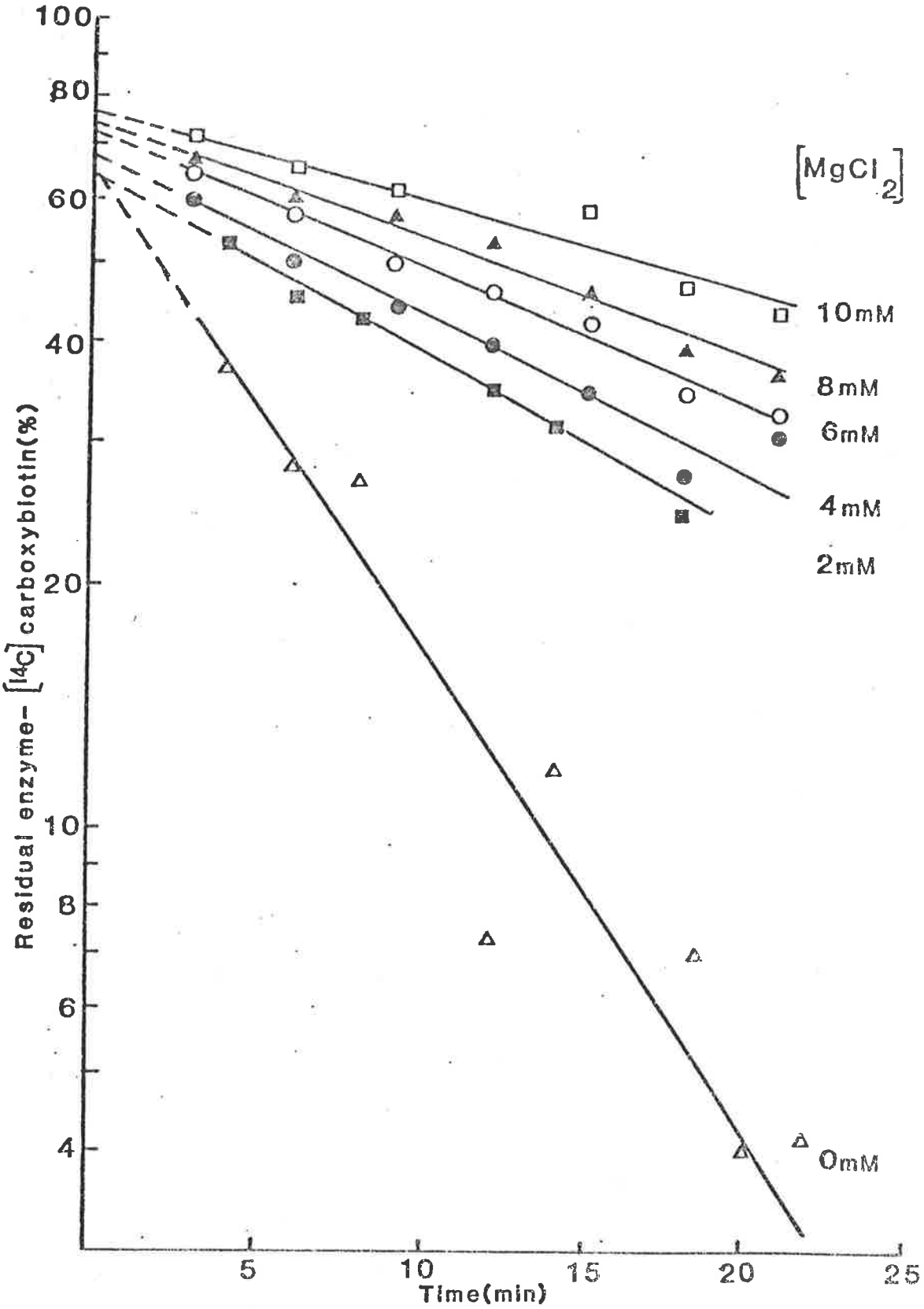


FIGURE 6.5 EFFECT OF ACETYL-CoA ON THE TIME COURSE OF TRANSLOCATION AND TRANSFER BY PYRUVATE CARBOXYLASE AT VARIOUS CONCENTRATIONS OF Mg^{2+}

The experimental conditions were the same as those described in Fig. 6.2 legend, except that 0.25 mM acetyl-CoA was present in all assay solutions.

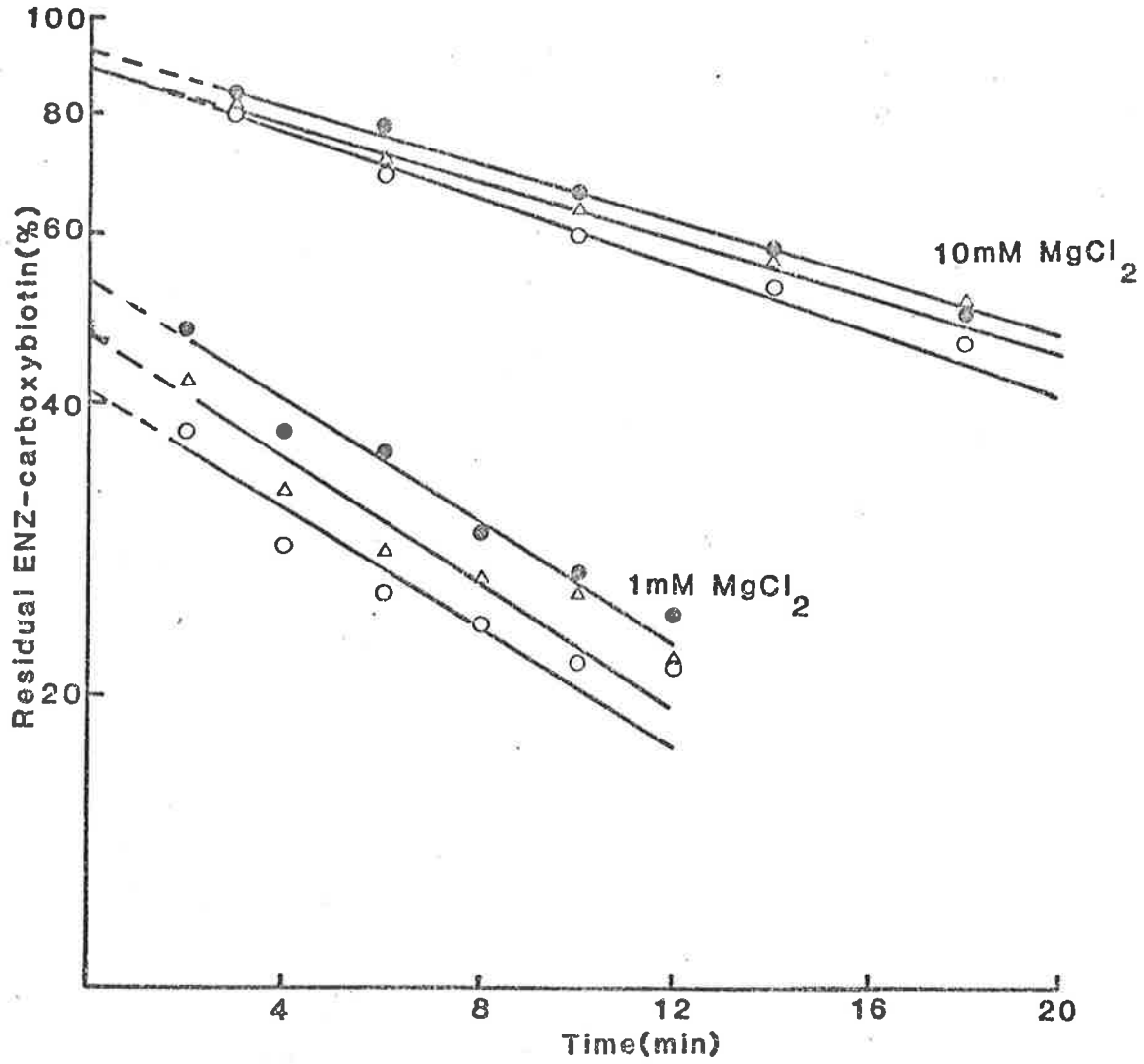


FIGURE 6.6

THE EFFECT OF 2-OXOBUTYRATE CONCENTRATION ON CARBOXYL TRANSFER

The experimental conditions were the same as those described in Fig. 6.1 except that the designated concentrations of $MgCl_2$ were included in the enzyme solution and the 2-oxobutyrate concentrations were 20 mM (●); 30 mM (Δ) or 40 mM (○). The lines were fitted using the GLIM program as described in the Methods section.

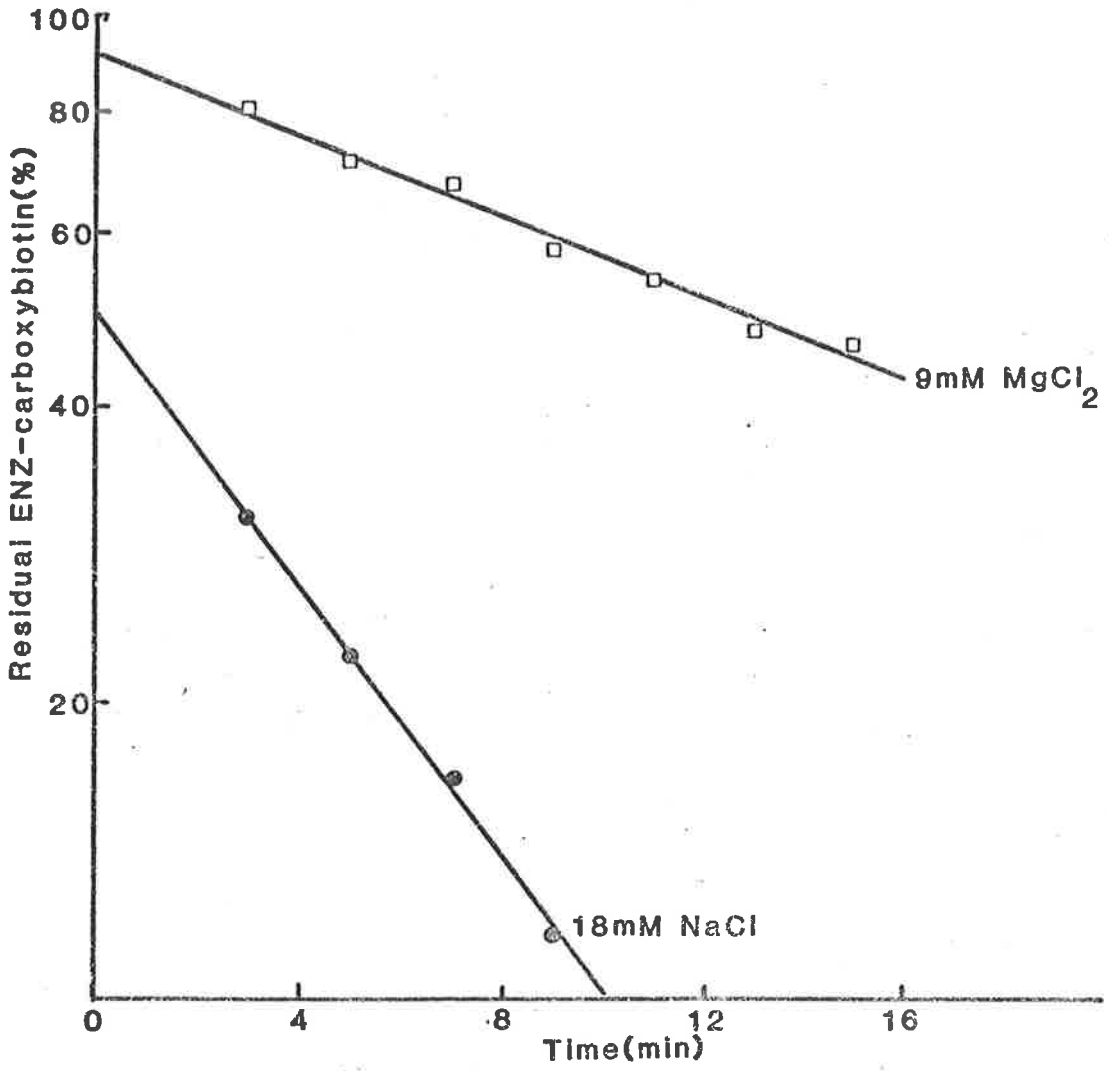


FIGURE 6.7

A COMPARISON OF THE EFFECT OF $MgCl_2$ AND NaCl ON THE TRANSCARBOXYLATION REACTION

The experimental conditions were the same as those described in Fig. 6.1 except that the pyruvate carboxylase was from chicken liver, and either 9 mM $MgCl_2$ (□) or 18 mM NaCl (●) was included in place of EDTA.

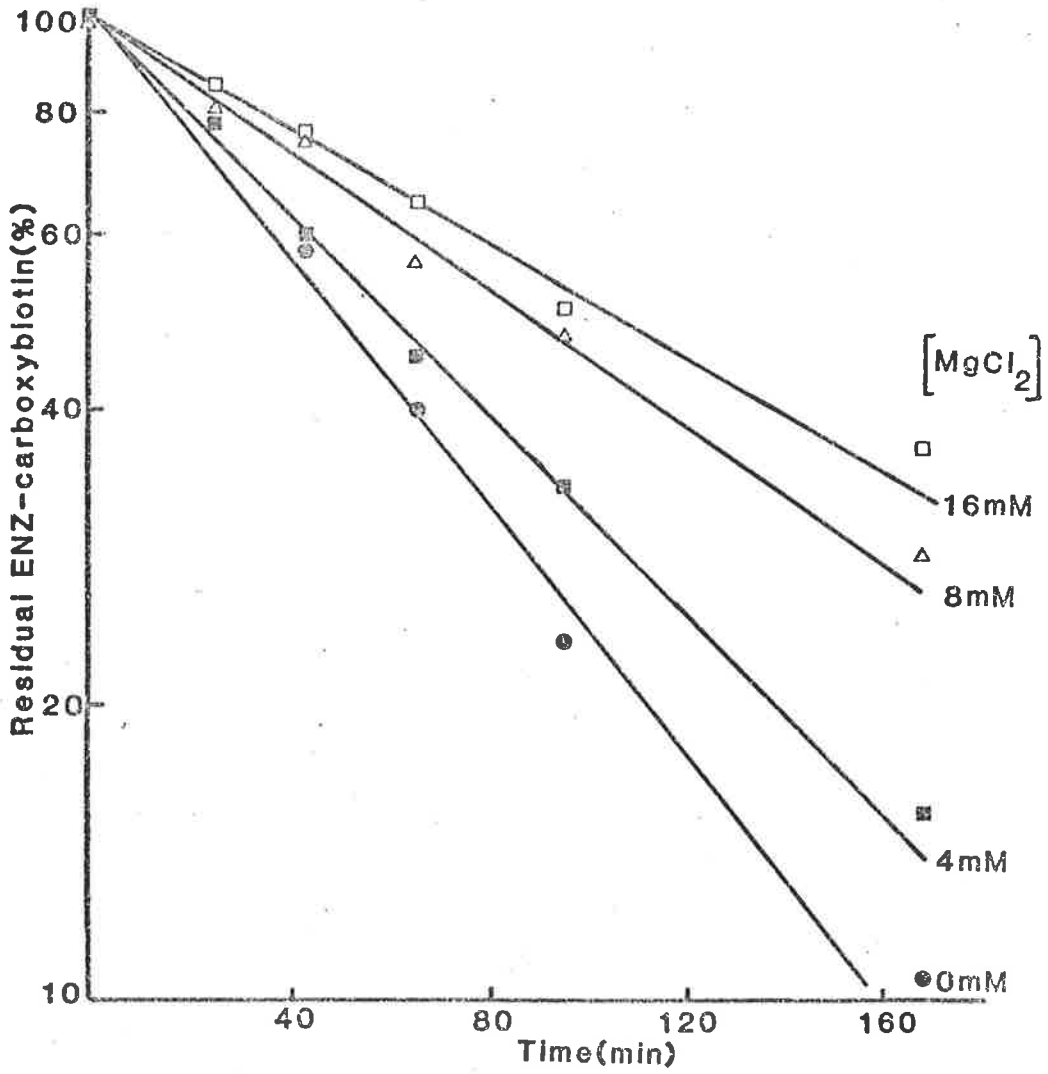


FIGURE 6.8

THE EFFECT OF Mg^{2+} ON THE STABILITY OF THE ISOLATED ENZ-[^{14}C]-CARBOXYBIOTIN COMPLEX

The isolated ENZ-[^{14}C]-carboxybiotin was incubated at 0°C in the presence of 100 mM N-ethylmorpholine-HCl buffer, pH 7.8, and the designated concentrations of $MgCl_2$. At time intervals 20 µl portions were removed and the amount of ENZ-[^{14}C]-carboxybiotin was determined by transferring the carboxyl group to pyruvate, as described in Section 6.2.3.

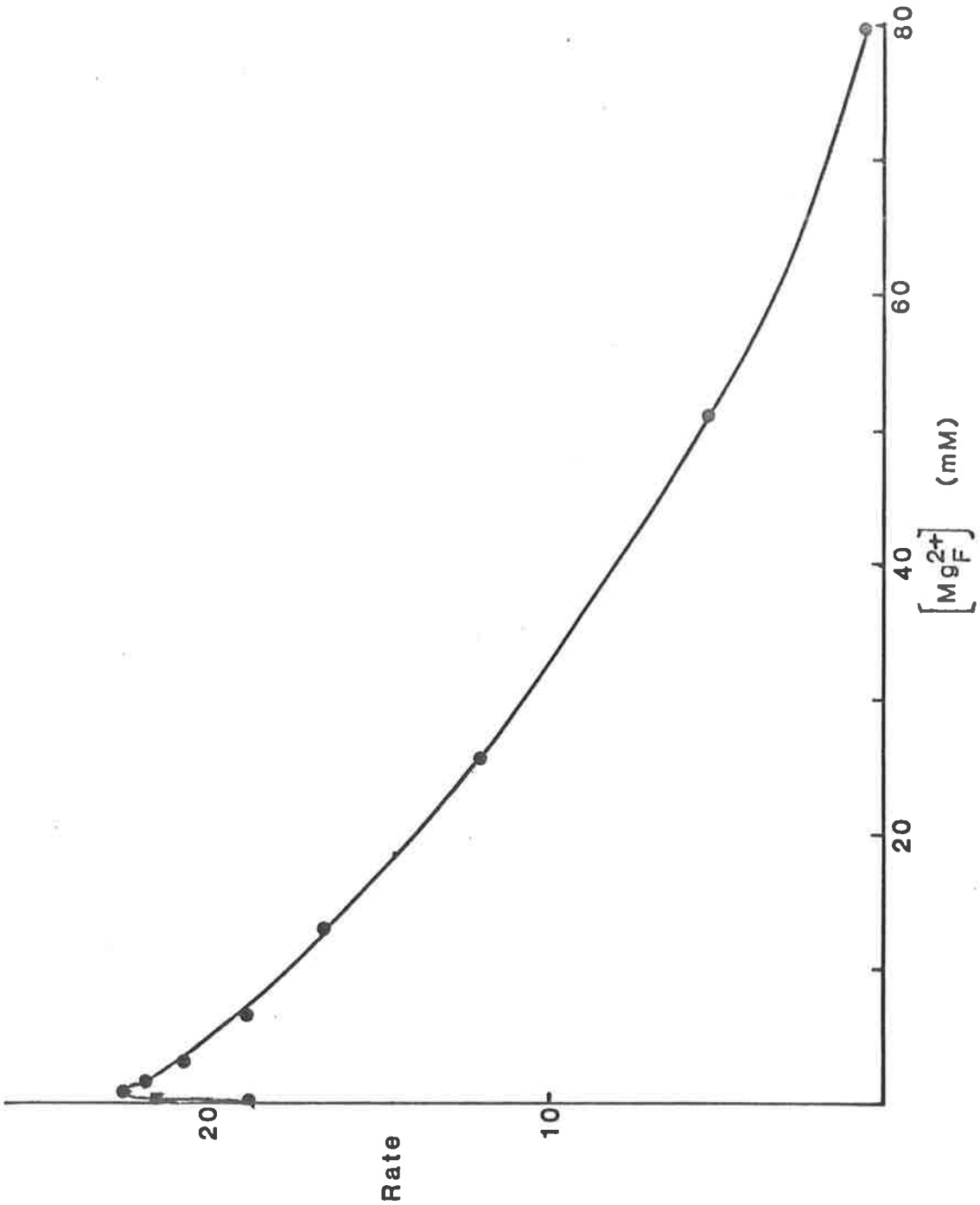


FIGURE 6.9

THE EFFECT OF Mg^{2+} ON OVERALL ACTIVITY

The reaction solution contained 100 mM Tris-Cl, pH 8.4, 2.5 mM ATP, 20 mM $NaHCO_3$, 10 mM pyruvate (sodium salt), 0.25 mM acetyl-CoA, 0.125 mM NADH, 5 units of malate dehydrogenase, 0.2 units of sheep liver pyruvate carboxylase (specific activity 17 units/mg) and varying concentrations of $MgCl_2$ to give the designated concentrations of free Mg^{2+}.

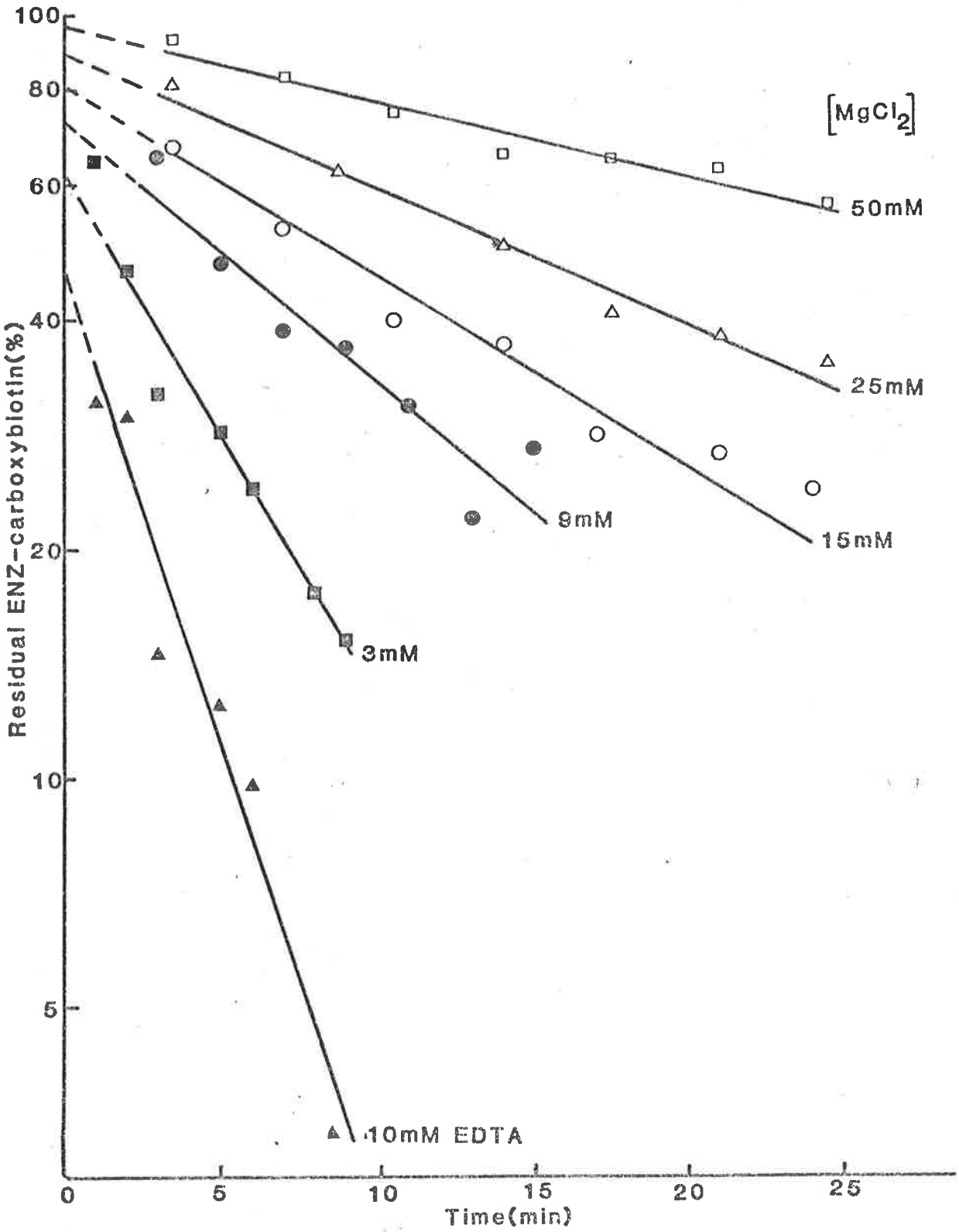


FIGURE 6.10 THE EFFECT OF Mg^{2+} ON THE TRANSLOCATION AND TRANSFER PROCESSES IN CHICKEN LIVER PYRUVATE CARBOXYLASE

The assay solution contained: 100 mM N-ethylmorpholine, pH 7.8; ENZ-[^{14}C]-carboxybiotin complex, 10^5 cpm/ml; 20 mM 2-oxo-butyrate; 0.25 mM acetyl-CoA; and 10 mM EDTA or the indicated concentration of $MgCl_2$.

CHAPTER 7

MECHANISM OF THE CARBOXYL GROUP TRANSFER REACTION

7.1 INTRODUCTION

Retey & Lynen in 1965 proposed that carboxyl transfer from carboxybiotin to its acceptor molecule as catalysed by propionyl-CoA carboxylase, occurred by a concerted reaction involving a six-membered ring complex (Fig. 7.1a). This proposal was based on their finding that carboxylation of propionyl-CoA occurs with retention of configuration, i.e., the carboxyl group is added to the same face of the substrate as that from which a proton is removed. The results of studies by Prescott & Rabinowitz (1968), involving deuterated and tritiated substrates, were consistent with the proposed concerted mechanism. They found that the rate of release of ^{3}H from [2-^{3}H]propionyl-CoA was the same as the rate of the overall reaction. Furthermore, tritium from $^{3}H_2O$ was not incorporated into propionyl-CoA in the absence of the reverse reaction.

The carboxylations catalysed by pyruvate carboxylase and transcarboxylase also occur with retention of configuration (Rose, 1970; Cheung *et al.*, 1975), and again, loss of a proton from the substrate does not occur in the absence of the other components of the overall reaction (Mildvan *et al.*, 1966; Rose *et al.*, 1976). At least on these criteria, it appears that the reactions catalysed by pyruvate carboxylase and transcarboxylase may occur by the same mechanism as that catalysed by propionyl-CoA carboxylase.

Another observation that is consistent with the concerted mechanism comes from the work of Rose *et al.*, (1976) who found that there is a small but significant amount of transfer of

hydrogen between the substrates of the two partial reactions catalysed by transcarboxylase: tritium from [3-^{3}H]-pyruvate to propionyl-CoA and tritium from [2-^{3}H]-propionyl-CoA to pyruvate. This transfer indicates that there is a proton carrier which functions between the two sub-sites of transcarboxylase. Rose *et al.* (1976) believe that the transfer is most likely to be via a base on the biotin carboxyl carrier protein and calculate that this base could be the 2'-O of biotin, despite the fact that an enol proton would rapidly equilibrate with the medium.

Carboxylation of pyruvate by both transcarboxylase and by pyruvate carboxylase occurs with a primary tritium isotope effect (Cheung *et al.*, 1975; Rose, 1970), i.e., when [3-$^{3}H_{1}$,$^{1}H_{2}$]pyruvate is the substrate, the ^{1}H is replaced by a carboxyl group faster than is the ^{3}H. [Strictly speaking, this is an intra-molecular primary tritium isotope effect, but the distinction does not matter here (c.f. Cheung & Walsh, 1976)]. On the other hand, carboxylation of propionyl-CoA by transcarboxylase and propionyl-CoA carboxylase occurs without a primary tritium isotope effect (Cheung *et al.*, 1975; Prescott & Rabinowitz, 1968). This difference could reflect the different natures of the transition states. Isotope effects are maximal when the proton being transferred is symmetrically located between the donor and acceptor in the activated complex (Westheimer, 1961). Perhaps steric hindrance by the C-3 methyl group of propionyl-CoA prevents a close approach of the proton-abstracting base, with the result that in the transition state the transferred proton is closer to the

propionyl-CoA C-2 than to the abstracting base. Alternatively, the lack of an isotope effect could be due to the abstraction of the proton in a rapid step, before the formation of the transition state. The fact that no exchange of substrate protons with solvent protons is observed in the absence of carboxylation (Prescott & Rabinowitz, 1964; Cheung *et al.*, 1975) does not quite rule out a pre-transition state proton abstraction, since the abstracted proton could conceivably be unable to exchange with solvent protons. In fact Stubbe *et al.*, (1980) have made observations which they interpret to indicate that proton abstraction from propionyl-CoA precedes carboxylation by both propionyl-CoA carboxylase and transcarboxylase. They found that both enzymes catalyse the elimination of HF from β-fluoropropionyl-CoA resulting in the formation of acrylyl-CoA, without the formation of detectable carboxylation products of β-fluoropropionyl-CoA. The mechanism they proposed (shown in Fig. 7.1b) to account for their observation is a two step mechanism in which a proton is first abstracted from the substrate by a base on the enzyme, leaving the substrate as a carbanion. The protonated base then forms a hydrogen bond with the ureido oxygen of carboxybiotin, thereby increasing the electrophilicity of the 1'-N-carboxyl group and so making it more susceptible to nucleophilic attack by the carbanion form of the acceptor substrate.

In both the concerted mechanism (Fig. 7.1a) and the carbanion mechanism (Fig. 7.1b) the proton removed from the substrate eventually becomes attached to the 2'-O of the

enol form of biotin. Thus both mechanisms can account for the observation made by Rose *et al.* (1976) that transcarboxylase transfers some hydrogen between the substrates of the two partial reactions catalysed by transcarboxylase.

A deficiency of the simple concerted mechanism shown in Fig. 7.1a is that model studies show that carboxybiotin, rather than being a reactive intermediate, is in fact relatively unreactive (Caplow, 1965; Caplow & Yager, 1967; Kohn, 1976; Visser & Kellogg, 1978; Stallings *et al.*, 1980). Of the three carboxy compounds that participate in the transcarboxylation reaction (oxaloacetate, carboxybiotin and methylmalonyl-CoA), carboxybiotin is the most stable (Wood *et al.*, 1963). Caplow (1965) has found that N-carboxyimidazolidone, a model of carboxybiotin, loses its carboxyl group by an intramolecular cyclic decarboxylation process and has no tendency to transfer it to a nucleophile. The enzymatic reaction is probably catalysed by more than the aligning of the acceptor substrate and the carboxybiotin. This is further supported by the crystal structure analyses of biotin (De Titta *et al.*, 1976) and 1'-N-methoxycarbonylbiotin methyl ester (Stallings *et al.*, 1980). The carboxylation of biotin decreases the polarization of the ureido carbonyl bond, decreasing its ability to remove a proton from a substrate. In proposing the concerted mechanism for pyruvate carboxylase, Mildvan *et al.* (1966) suggested that transannular interaction between the carbonyl group and the sulphur atom of biotin might contribute to polarization of the carbonyl group. This would assist the removal of a proton from the substrate molecule. However, Bowen *et*

al. (1968) have since shown that such an interaction could have only a negligibly small effect. Mildvan *et al.* (1966) also proposed that the tightly bound Mn^{2+} ion co-ordinated with the carbonyl oxygen of pyruvate and the carboxyl group of 1'-N-carboxybiotin, thereby facilitating the departure of a proton from the methyl group of pyruvate and assisting the formation of a carbonium ion from carboxybiotin. However, Fung *et al.* (1973) have since shown that pyruvate binds too far away from the Mn^{2+} to allow direct interaction. Fung *et al.* (1974) and Mildvan (1974) have subsequently suggested that the bound metal ion in pyruvate carboxylase and transcarboxylase promotes the acidity of an inner sphere water ligand which forms a hydrogen bond with the carbonyl oxygen of pyruvate, thereby assisting proton abstraction from the methyl group. Fung *et al.* (1976) proposed two alternative concerted mechanisms which incorporate this suggestion.

The experiments described in this chapter examine further the mechanism of carboxyl transfer reactions catalysed by biotin-dependent enzymes. A mechanism is proposed which is consistent with the observations that have been made on several different biotin-dependent enzymes. Although such a mechanism is necessarily speculative at this stage, it is important to the proposal that the biotin-dependent enzymes constitute an evolutionary family that they have similar catalytic mechanisms.

7.2 METHODS

7.2.1 Measuring ATP hydrolysis

The reaction mixture contained 2.16 mM [γ-^{32}P]ATP (4 mCi/mmol), 7 mM $MgCl_2$, 25 mM $NaHCO_3$, 2 mM DTE, 100 mM KCl, 0.1 M N-ethylmorpholine-HCl buffer, pH 8.0, 0.002 units of propionyl-CoA carboxylase and various concentrations of propionyl-CoA in a volume of 0.2 ml. After 4 min at 30°C the reaction was stopped by the addition of 50 µl of concentrated formic acid. Aliquots of the solution were applied to polyethyleneimine thin layer plates and the chromatographs developed using 0.08 M NH_4HCO_3 as the developing solvent. These were cut into 0.5 cm strips and the proportion of [^{32}P] as Pi was determined by liquid scintillation counting.

[γ-^{32}P]ATP was a gift from Dr. R.H. Symons and was purified by the method of Symons (1977).

7.2.2 Measurement of ^{3}H release from pyruvate

The conditions were those of the normal spectrophotometric assay, except that various concentrations of [3-^{3}H]-pyruvate were used. The reaction was initiated by the addition of pyruvate carboxylase (specific activity 15). At 1 min intervals, 50 µl portions of the reaction solution were removed from the spectrophotometer cuvette and added to 20 µl of 2 M semicarbazide-hydrochloride. After 1.25 min the solution was diluted with 180 µl of water and kept in a sealed Eppendorf tube until the time-course was complete. The samples were then dried under vacuum, and the water from each trapped in a cold trap. The radio-

activity in a 200 µl portion of the water removed from each was counted. Controls showed that the water can be reproducibly and quantitatively isolated by this method.

7.3 RESULTS

7.3.1 A test of the carbanion mechanism

In the carbanion mechanism proposed by Stubbe *et al.* (1980) (Fig. 7.1b) a protonated base forms a hydrogen bond with the ureido oxygen of carboxybiotin, thereby increasing the electrophilicity of the 1'-N-carboxyl group and so making it more prone to hydrolysis if a suitable acceptor substrate is not present to make a nucleophilic attack on the carboxyl group. After discharging its carboxyl group the resulting enol biotin will rapidly tautomerise to the ureido form (Glasel, 1966), liberating the abstracted proton to the solvent. Such a situation apparently arises when β-fluoropropionyl-CoA is used as a substrate for propionyl-CoA carboxylase or transcarboxylase (Stubbe *et al.*, 1980), or when the pyruvate carboxylase reaction occurs in the presence of low concentrations of pyruvate (i.e., in the abortive decarboxylation pathway), (Easterbrook-Smith *et al.*, 1976a). Thus if the carbanion mechanism in Fig. 7.1b applies to pyruvate carboxylase, we would expect the rate of proton abstraction to equal the rate of ATP hydrolysis (i.e., the sum of the rate of oxaloacetate production and the rate of carboxybiotin hydrolysis), and, especially at low concentrations of pyruvate, to exceed the rate of oxaloacetate production.

The rate of liberation of ^{3}H from [3-^{3}H]pyruvate and the rate of oxaloacetate production were measured simultaneously over a range of pyruvate concentrations (Fig. 7.2). If the reaction occurred by the carbanion mechanism described above, then, from the results of Easterbrook-Smith *et al.* (1976a), the ratio of the rate of tritium release to the rate of oxaloacetate production should have decreased by 1.6 - fold when the pyruvate concentration was increased from 0.5 mM to 2 mM. Figure 7.2 shows there was no change at all in this ratio and so the carbanion mechanism apparently does not apply to pyruvate carboxylase. (The absolute rates of proton abstraction and oxaloacetate production were not compared as [3-^{3}H]pyruvate is not as good a substrate as [3-^{1}H]pyruvate, i.e., there is a tritium isotope effect).

One might argue that the hydrolysis of carboxybiotin could occur while the pyruvate carbanion is still bound to the enzyme, if the active site is "loose" and allows a water molecule to intervene between the pyruvate and the carboxybiotin. This might allow the abstracted proton to return to the pyruvate C-3 after the carboxybiotin has been hydrolysed. However, if this were the case, the ratio of ATP hydrolysis to oxaloacetate production should be independent of the pyruvate concentration, whereas the ratio changes with pyruvate concentration (Easterbrook-Smith *et al.*, 1976a).

Further evidence against the "loose active site" proposal was obtained by examining the effect of certain pyruvate analogues on the stability of the carboxybiotin to

hydrolysis. Glyoxylate and oxamate, the structures of which are shown in Fig. 7.3, cannot participate in the carbanion mechanism (Fig. 7.1b) because they cannot form a carbanion. Nevertheless, they stimulate the rate of hydrolysis of the isolated ENZ-[^{14}C]-carboxybiotin complex (Fig. 7.4) and they increase the curvature of the double reciprocal plot with varied pyruvate concentration (Fig. 7.5), indicating an increased flux through the abortive hydrolytic pathway. Hydroxypyruvate, which can potentially form a carbanion, but is not a substrate of pyruvate carboxylase, has similar effects on the stability of the carboxybiotin to those of glyoxylate and oxamate (Figs. 7.4 and 7.5). Thus it seems that glyoxylate, oxamate and hydroxypyruvate can act as signals in the same way that pyruvate and 2-oxobutyrate do, inducing carboxybiotin to bind at the second sub-site, where it is unstable (see Chapter 6). The structural properties of the acceptor substrate molecule that influence the translocation of carboxybiotin were studied and are discussed in the next section.

7.3.2 The binding of pyruvate to pyruvate carboxylase

During the course of the overall reaction catalysed by pyruvate carboxylase, it is necessary for the carboxybiotin to be released from the first sub-site and moved to the second sub-site. Here it transfers its activated carboxyl group to the acceptor molecule. In this study a number of compounds possessing structural features resembling pyruvate were examined to determine which features of the pyruvate molecule affect the binding of

pyruvate to the enzyme and the translocation of carboxybiotin from the first sub-site to the second sub-site. The compounds used fall into three groups: (a) those that are substrates for pyruvate carboxylase (i.e., pyruvate, fluoropyruvate, 2-oxobutyrate), (b) those that are inhibitors (hydroxypyruvate, oxamate, glyoxylate) and (c) those that have no effect on the enzyme (propionate, lactate, acetaldehyde). The structures of these compounds are shown in Fig. 7.3.

When either lactate, propionate or acetaldehyde was substituted for pyruvate in the radiochemical assay, no fixation of $H^{14}CO_3^-$ was detected. Furthermore, none of these compounds inhibit the overall activity of the enzyme (Table 7.1). In addition, since lactate, propionate and acetaldehyde do not affect either the stability of the ENZ-carboxybiotin complex (Fig. 7.6) or the transfer of the carboxyl group to 2-oxobutyrate (Fig. 7.7), we can conclude that these compounds do not perturb the equilibrium between State I and State II (which have been defined in Section 6.3.1). Thus, both the carboxyl and keto group of pyruvate appear to be essential both for binding and initiating the transfer process.

While the transition from State I to State II is slow enough to measure with 2-oxobutyrate as the acceptor substrate, the transition is too fast to measure under similar conditions if either pyruvate or fluoropyruvate is used as acceptor (Fig. 7.8). This means that both pyruvate and fluoropyruvate initiate the release of the carboxybiotin from the first sub-site, but the addition of a methyl group

at the C-3 position (as in 2-oxobutyrate) drastically reduces the efficiency of the releasing process.

Hydroxypyruvate, oxamate and glyoxylate also differ from pyruvate at the C-3 position, but no carboxylation of these compounds could be detected when they replaced pyruvate in the radiochemical assay. However, since they act as inhibitors, these compounds do bind to the enzyme (Fig. 7.5). Thus the methyl group of pyruvate does not appear to be essential for binding. However, it is difficult to determine whether or not the inhibition of the overall reaction by hydroxypyruvate, glyoxylate and oxamate is competitive with respect to pyruvate. This is because double reciprocal plots with pyruvate as the varied substrate are concave downward, especially in the presence of the inhibitors used here. Nevertheless, it is quite possible that the double reciprocal curves of Fig. 7.5 intersect on the ordinate. Hence, in view of the similarity of the inhibitor compounds to pyruvate, it is reasonable to assume that they bind to the enzyme at the pyruvate binding site.

In Section 7.3.1 it was shown that glyoxylate, oxamate and hydroxypyruvate act as efficient translocation signals, attracting carboxybiotin into the second subsite, but since they are not carboxylated (Fig. 7.4) the complex undergoes hydrolysis. It should be noted that the rate of translocation and hydrolysis of the complex in the presence of these compounds is much faster than the rate of translocation in the presence of 2-oxobutyrate. The behaviour of these compounds is consistent with the conclusion that the carboxyl and keto groups of pyruvate are necessary for binding

and initiating the translocation of the carboxybiotin. However, the fact that glyoxylate acts as an efficient signal suggests that the methyl group is not required for these processes.

7.3.3 The second partial reaction of propionyl-CoA carboxylase

It is not known whether the various biotin-dependent enzymes all use a similar mechanism to catalyse the transfer of the carboxyl group from carboxybiotin to the acceptor substrate. In view of the presence of a hydrolytic leak in the pyruvate carboxylase reaction, it is of interest to determine whether such a leak occurs during the reaction catalysed by propionyl-CoA carboxylase.

The rate of the propionyl-CoA carboxylase reaction was measured both in terms of methylmalonyl-CoA produced and orthophosphate liberated. The data obtained are presented in Fig. 7.9 in the form of a double reciptoral plots. It can be seen that the rate of phosphate release is the same as the rate of methylmalonyl-CoA production over a 67-fold range of propionyl-CoA concentrations. The contrast between the constant stoichiometry of propionyl-CoA carboxylase and the varying stoichiometry of pyruvate carboxylase is evident in Fig. 7.10 where for each enzyme the ratio of Pi released to CO_2 fixed is plotted against acceptor substrate concentration. The reason for the propionyl-CoA carboxylase stoichiometry being slightly less than 1:1 is probably a small error in the determination of the specific activity of $NaH^{14}CO_3$ used. (All solutions at the pH of the assay

solution contain non-radioactive endogenous HCO_3^-).

The absence of a hydrolytic pathway is further demonstrated by the linearity of the double reciprocal plot with varying propionyl-CoA concentration (Figs. 7.9 and 7.11). A reaction pathway with alternative possible products (such as HCO_3^- and methylmalonyl-CoA) will generally yield a non-linear double reciprocal plot (Easterbrook-Smith, 1977).

The presence of an abortive hydrolytic pathway in the pyruvate carboxylase reaction but not the propionyl-CoA carboxylase reaction could result from the enzymes having different catalytic mechanisms. However, it can, like the different tritium isotope effects, be rationalized in the hypothesis of common mechanisms. Cheung & Walsh (1976) have shown that when pyruvate binds to the carboxybiotin form of pyruvate carboxylase, it is just as likely to dissociate without having been carboxylated as it is to be converted to oxaloacetate. It is on the occasions that pyruvate dissociates that hydrolysis of carboxybiotin can occur. On the other hand, propionyl-CoA may be carboxylated every time it binds to enzyme-carboxybiotin. Being a much larger molecule, it has more functional groups available for binding, and so its dissociation from the enzyme may be slower. This proposal could be tested by the isotope trapping method (Rose, 1980).

7.4 DISCUSSION

The results presented in this chapter indicate that neither the simple concerted mechanism shown in Fig. 7.1a

nor the carbanion mechanism shown in Fig. 7.1b apply to pyruvate carboxylase. The carbanion mechanism cannot account for the lack of proton abstraction in the abortive hydrolytic pathway, or for the stimulation of carboxybiotin hydrolysis by glyoxylate and oxamate. The concerted mechanism cannot account for the abortive hydrolytic pathway at all, in the presence of either low concentrations of pyruvate or of analogues such as oxamate and glyoxylate. However, as propionyl-CoA carboxylase does not abortively decarboxylate in the presence of propionyl-CoA, the carbanion mechanism cannot be ruled out for this enzyme.

We can account for all of the observations discussed in this chapter if the carboxylation reactions catalysed by biotin-dependent enzymes occur via the mechanism shown in Fig. 7.12. The sequence of events in this mechanism is as follows;- The binding of the acceptor substrate at the second sub-site induces a conformational change in the enzyme with the result that the 1'-N-carboxybiotin translocates and binds at the second sub-site. In the second sub-site a base is positioned adjacent to the 3'-N of the carboxybiotin and abstracts a proton from the 3'-N, producing an enolate anion of carboxybiotin. A cyclic concerted reaction similar to the one originally proposed by Rétey & Lynen (1965) then occurs, except that it is the enolate anion of carboxybiotin that reacts, rather than the ureido form. Once the carboxylated product is formed, it dissociates from the enzyme and the enol-biotin returns to the first sub-site, in most cases tautomerising to the uredio form on the way.

As the step in which the product is formed is concerted, the proposed mechanism is consistent with the observations of retention of configuration, lack of exchange between substrate and water protons when the enzyme is not carboxylated, and transfer of tritium between the substrates of transcarboxylase.

The abortive hydrolysis of carboxybiotin at low concentrations of pyruvate could occur if the pyruvate molecule, having induced the carboxybiotin to bind at the second sub-site, dissociates from the enzyme. This would allow a water molecule access to the enolate anion of carboxybiotin and a cyclic reaction would occur with water as the substrate instead of pyruvate (Fig. 7.13). Pyruvate analogues such as oxamate and glyoxylate would cause decarboxylation in the same way. We could consider the signalling of carboxybiotin to the second sub-site by the binding of pyruvate to be equivalent to an induced fit mechanism.

The proposed mechanism also accounts for the observation made by Stubbe *et al.* (Stubbe & Abeles, 1977; Stubbe *et al.*, 1980) that propionyl-CoA carboxylase and transcarboxylase catalyse the elimination of F^- from β-fluoropropionyl-CoA with concomitant decarboxylation of carboxybiotin. The proposed pathway of this reaction is shown in Fig. 7.14. The binding of β-fluoropropionyl-CoA to the enzyme induces carboxybiotin to bind at the propionyl-CoA binding sub-site, where the enolate anion of carboxybiotin is formed. The anion abstracts a proton from C-2 of the substrate analogue, as it would in the normal reaction, but instead of the C-2 of the substrate simultaneously attacking the 1'-N-carboxyl

group of carboxybiotin, fluoride is eliminated from the fluoropropionyl-CoA. [Stubbe *et al.* (1980) have suggested several reasons why fluoride elimination occurs in preference to carboxylation.] The net result is the formation of F^- and acrylyl-CoA from β-fluoropropionyl-CoA and enol carboxybiotin from the enolate anion. In the next steps, the acrylyl-CoA dissociates from the enzyme and the carboxybiotin returns to the ureido form. Water now has access to the carboxybiotin, and hydrolysis can occur in the manner already described. Fluoride elimination is only catalysed by enzyme that is in the carboxybiotin form (Stubbe *et al.*, 1980). This is not inconsistent with the proposed mechanism as the appropriate alignment of substrate and biotin in the active site may occur only when one of them is carboxylated. It is also possible that there is room for one or more water molecules to bind in place of the carboxyl group. The removal by the enzyme of a proton from the 3'-N of biotin would then promote enolization of the biotin, instead of enolate ion formation.

TABLE 7.1

PYRUVATE ANALOGUES IN THE OVERALL REACTION

ANALOGUE	V_{max} (a)	% INHIBITION (b)
lactate	0	0 (100 mM)
propionate	0	0 (100 mM)
acetaldehyde	0	6% (100 mM)
glyoxylate	0	73% (10 mM)
hydroxypyruvate	0	54% (10 mM)
oxamate	0	91% (10 mM)
2-oxobutyrate	4% (c)	-
fluoropyruate	5% (c)	-

(a) The V_{max} for the analogue as substrate is expressed as a % of the V_{max} with pyruvate as substrate

(b) The conditions are those of Fig. 7.5, with 2 mM pyruvate. The analogue concentration is shown in brackets.

(c) From Easterbrook-Smith *et al.* (1978).

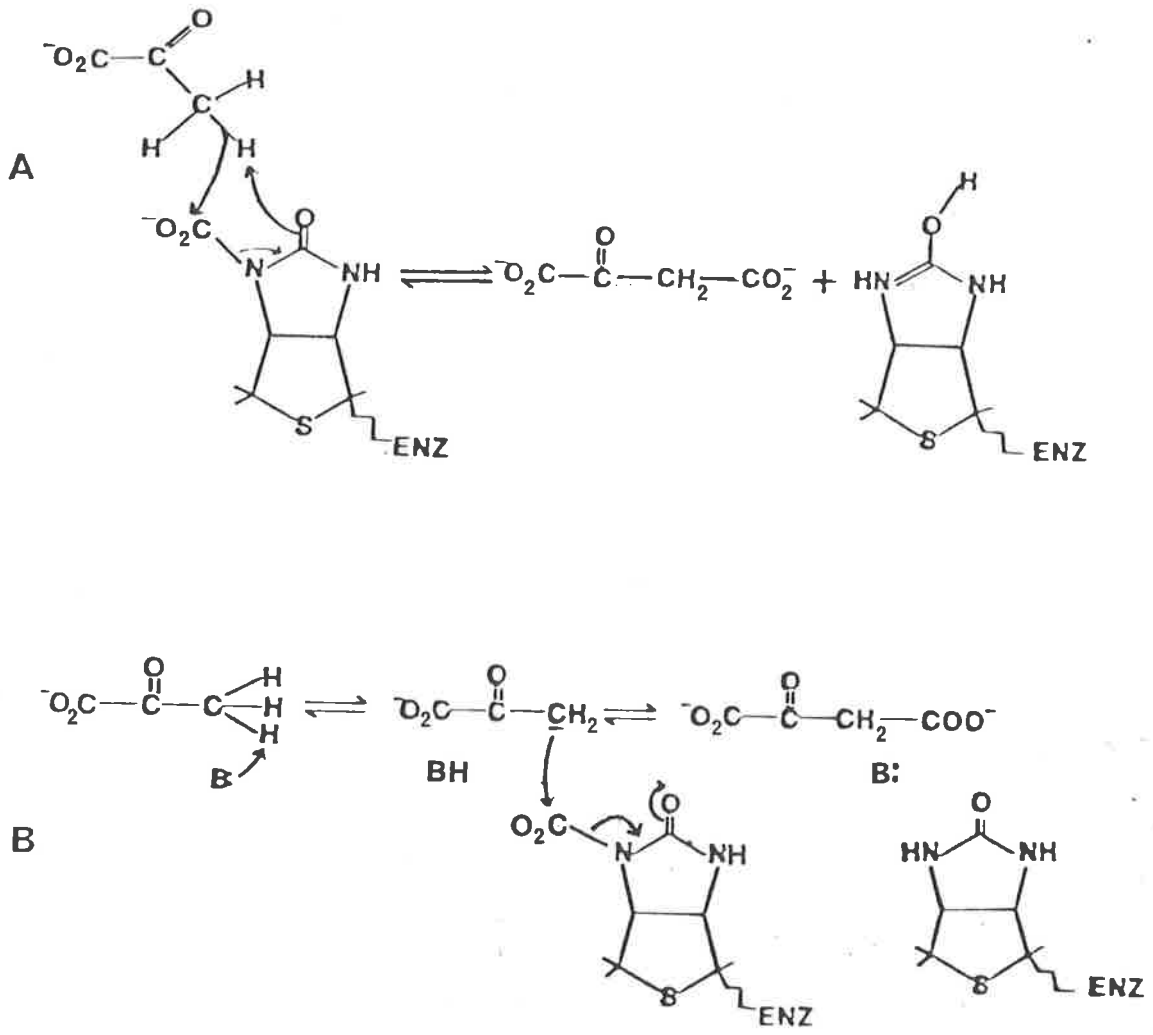


FIGURE 7.1

PREVIOUSLY PROPOSED MECHANISMS OF CARBOXYL TRANSFER

A. The concerted mechanism proposed originally by Rétey & Lynen (1965).

B. The carbanion mechanism proposed by Stubbe & Abeles (1977: Stubbe *et al.*, 1980).

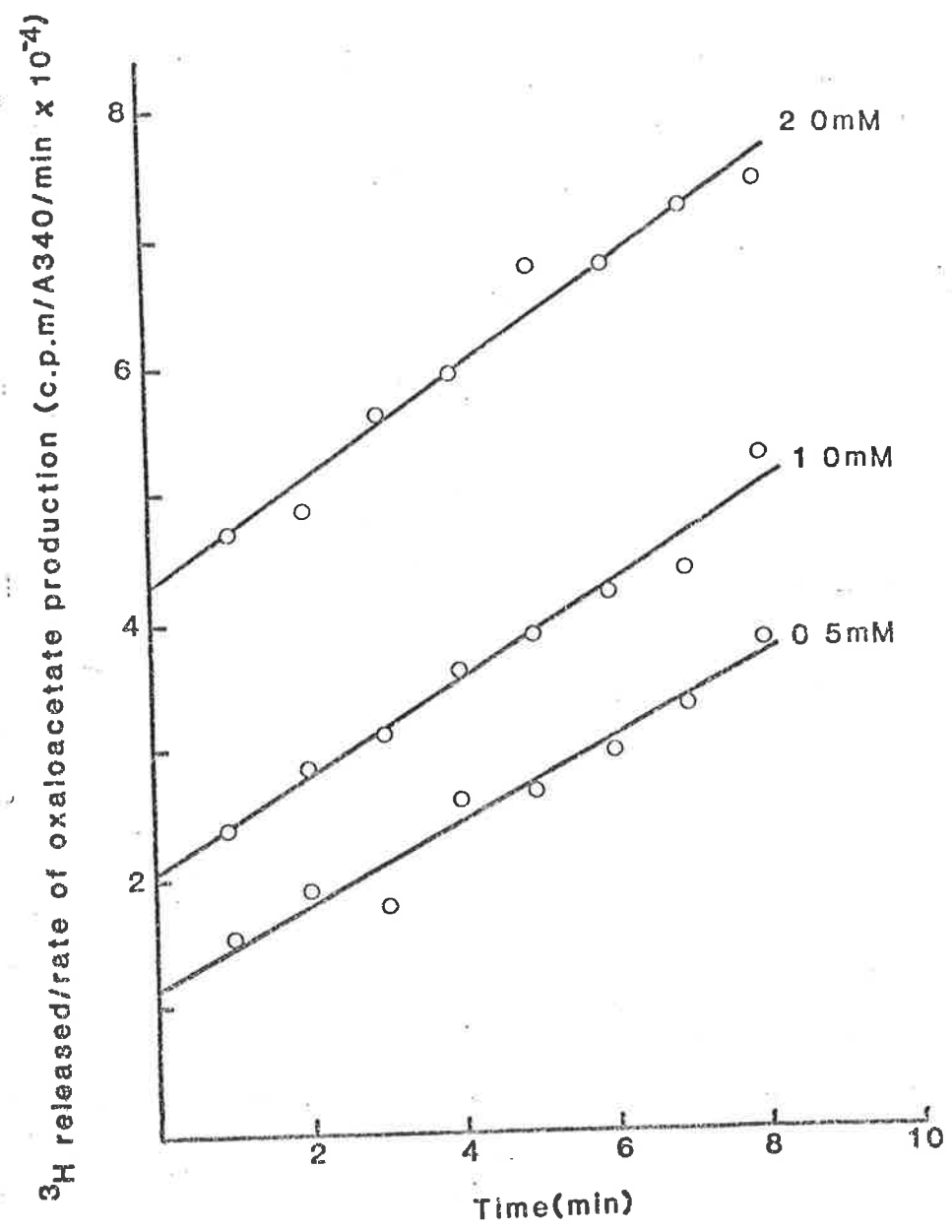


FIGURE 7.2

THE RATES OF ^{3}H RELEASE AND OXALOACETATE PRODUCTION AT VARIOUS CONCENTRATIONS OF PYRUVATE

The rate of oxaloacetate production was measured spectrophotometrically, as described in Section 2.2.7, except that the designated concentrations of [^{3}H]pyruvate were used. The measurement of ^{3}H released from pyruvate is described in Section 7.2.2. The lines do not pass through the origin because [3-^{3}H]pyruvate enolizes on storage. The lines were fitted by simple linear regression and the slopes were not significantly different (analysis of variance, P>5%).

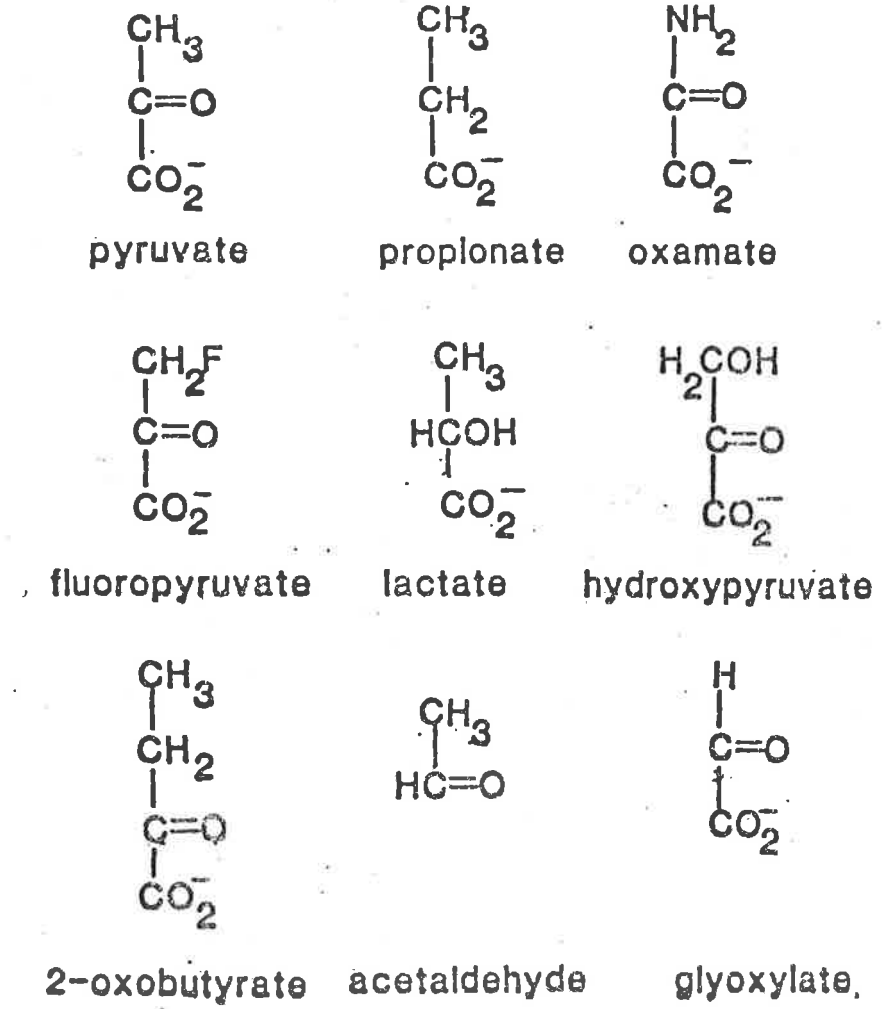


FIGURE 7.3

THE STRUCTURES OF ANALOGUES OF PYRUVATE USED IN THIS STUDY.

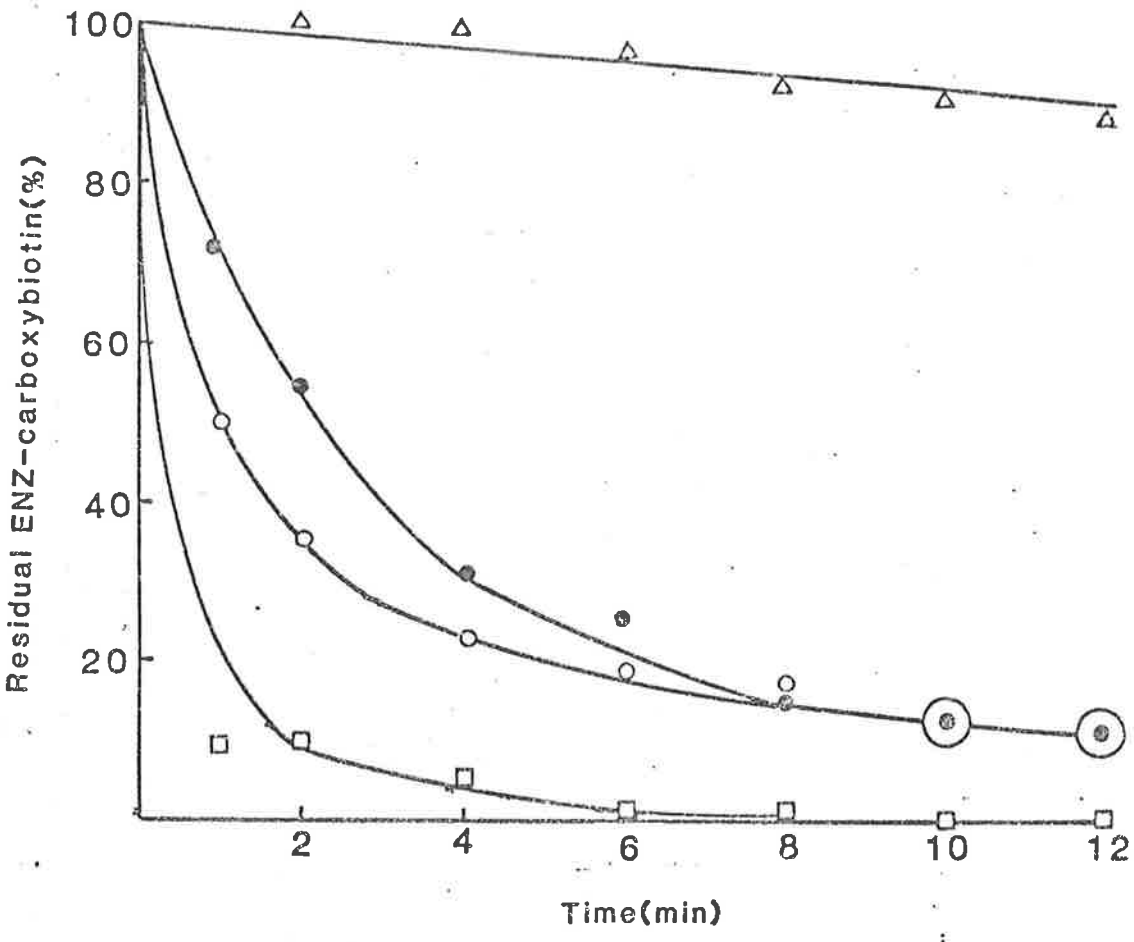


FIGURE 7.4

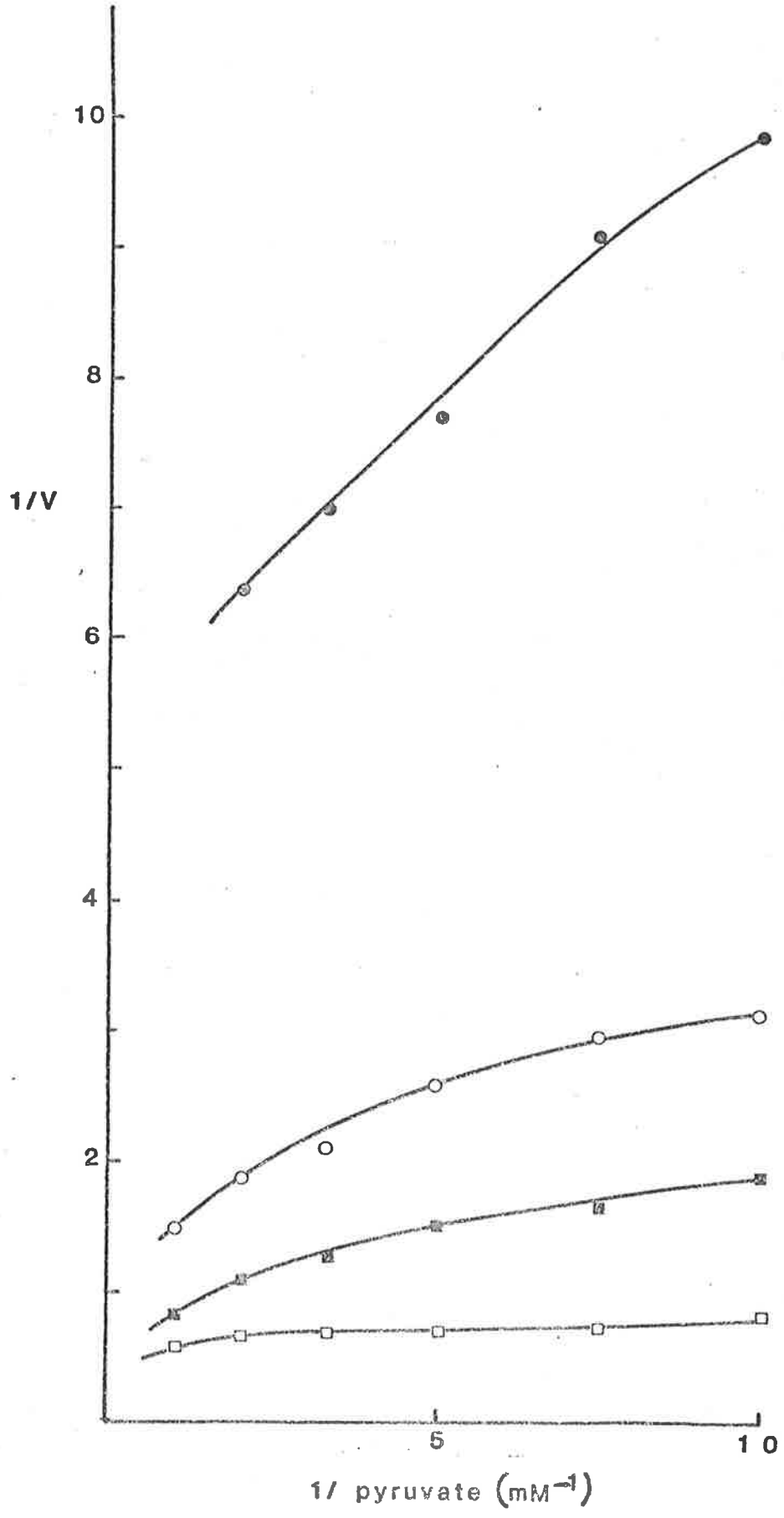
EFFECT OF A-OXO ACID DERIVATIVES ON THE STABILITY OF THE [^{14}C]CARBOXYBIOTIN COMPLEX OF PYRUVATE CARBOXYLASE.

The assay solutions contained: 100 mM N-ethylmorpholine/HCl buffer, pH 7.8, 6 mM $MgCl_2$ and enzyme-[^{14}C]carboxybiotin (3 x 10^4 cpm/ml). The reaction was started by the addition of 10 mM hydroxypyruvate (□), 10 mM glyoxylate (●) or 10 mM oxamate (○). Water was added to the control (△). The residual enzyme-[^{14}C]carboxybiotin complex was measured at the times indicated by transfer to pyruvate as described in the Methods and materials section.

FIGURE 7.5

INHIBITION OF OVERALL ACTIVITY BY VARIOUS ANALOGUES OF PYRUVATE

The assay solution contained 2.5 mM ATP, 7 mM $MgCl_2$, 20 mM $NaHCO_3$, 0.25 mM acetyl-CoA, 0.25 mM NADH, 0.2 units pyruvate carboxylase, 10 units malic dehydrogenase, 100 mM N-ethyl-morpholine-HCl buffer, pH 8.4, and the indicated concentrations of pyruvate in a 1 ml volume at 30°C. Additions were 10 mM oxamate (●), 10 mM glyoxylate (○), 10 mM hydroxypyruvate (■) or no addition (□). The rate was determined by the absorbance change at 340 nm.


10
8
6
4
2
1/V
5
10
1/ pyruvate (mM⁻¹)

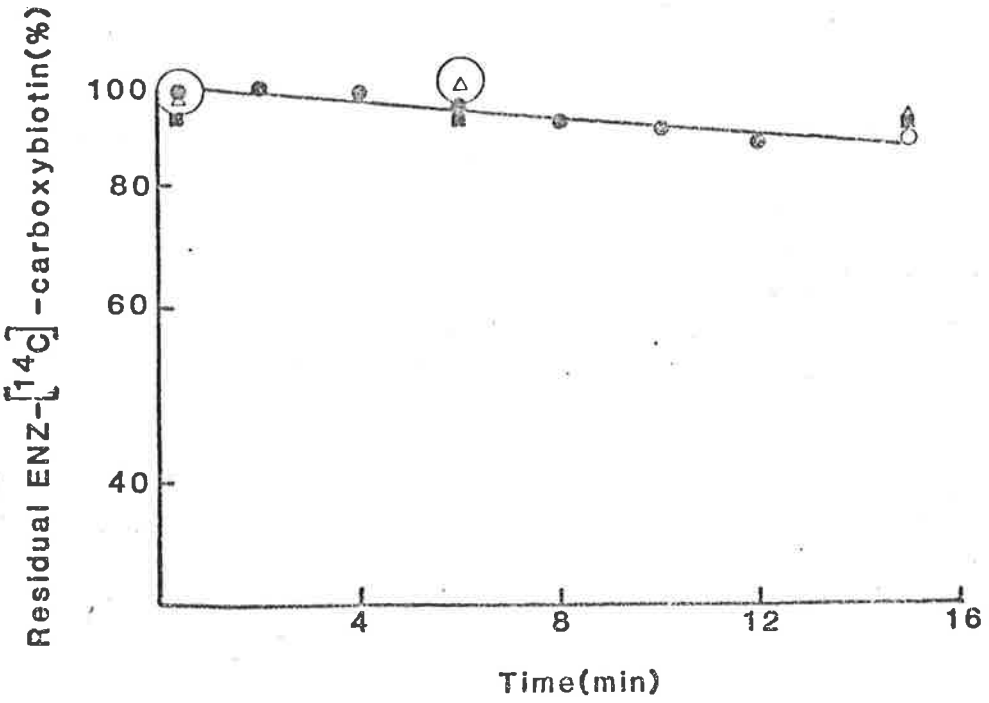


FIGURE 7.6

THE EFFECT OF CERTAIN ANALOGUES OF PYRUVATE ON THE HYDROLYSIS OF ENZ-CARBOXYBIOTIN

The solution contained ENZ-[^{14}C]carboxybiotin, 10 mM N-ethylmorpholine-HCl buffer, pH 7.8, and 6 mM $MgCl_2$. Additions were 10 mM propionate (■), 10 mM lactate○ 10 mM acetaldehyde△ or no additions (●). The amount of ENZ-[^{14}C]carboxybiotin was measured as described in Section 6.2.3.

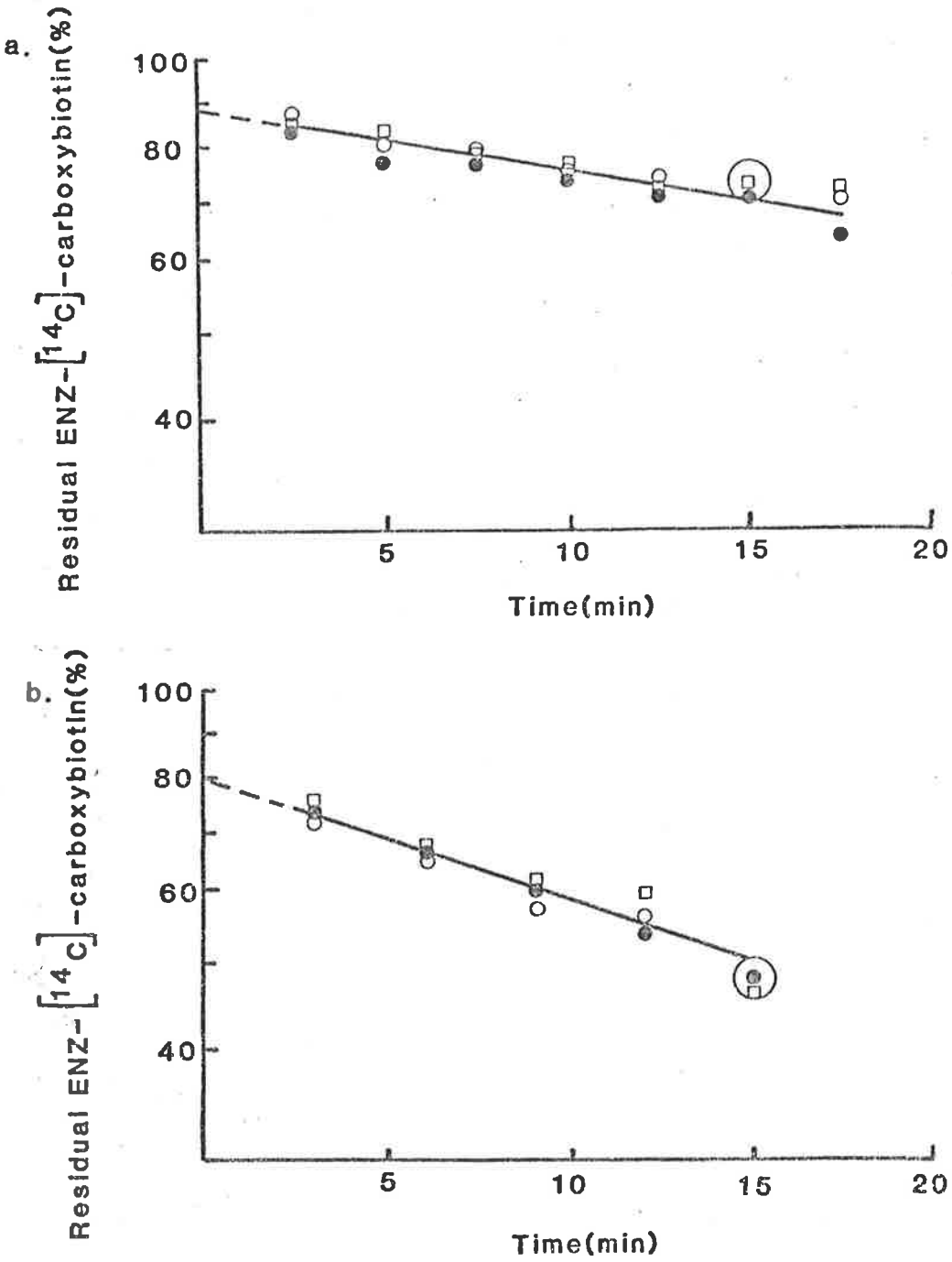


FIGURE 7.7

CARBOXYLATION OF 2-OXOBUTYRATE BY ENZ-CARBOXYBIOTIN IN THE PRESENCE OF CERTAIN ANALOGUE OF PYRUVATE

A. The reaction solution contained ENZ-[^{14}C]-carboxybiotin, 20 mM 2-oxobutyrate, 10 mM $MgCl_2$, 100 mM N-ethylmorpholine-HCl buffer, pH 7.8. Additions were 2 mM acetaldehyde (□), 2 mM lactate (○) or no addition (●).

B. The reaction solution contained ENZ-[^{14}C]-carboxybiotin, 20 mM 2-oxobutyrate, 6 mM $MgCl_2$, 100 mM N-ethylmorpholine-HCl buffer, pH 7.8. Additions were 2 mM propionate (□), 2 mM lactate (○) or no addition (●).

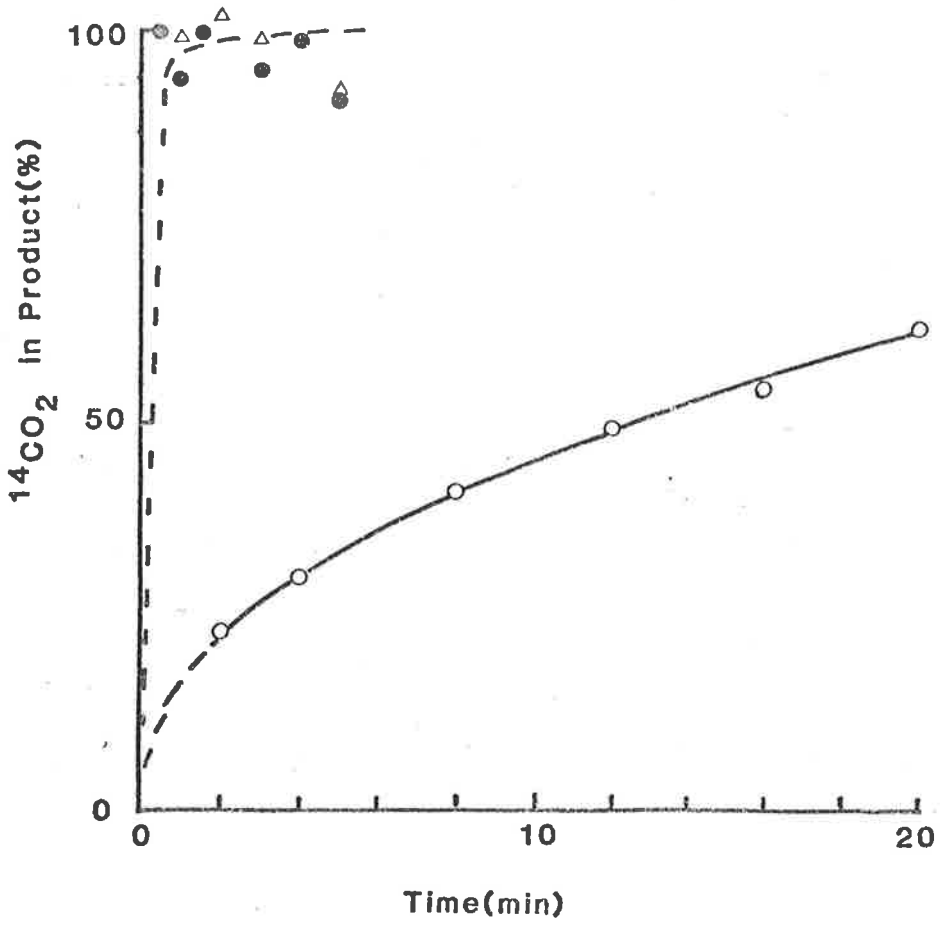


FIGURE 7.8

TIME COURSE OF TRANSCARBOXYLATION FROM ENZ-CARBOXYBIOTIN TO PYRUVATE, FLUOROPYRUVATE AND 2-OXOBUTYRATE

The experimental conditions were the same as those described in Fig. 6.1 except that 6 mM $MgCl_2$ was included in the enzyme solution and the carboxyl acceptor was 10 mM pyruvate (△), 20 mM fluoropyruvate (●) or 20 mM 2-oxobutyrate (○).

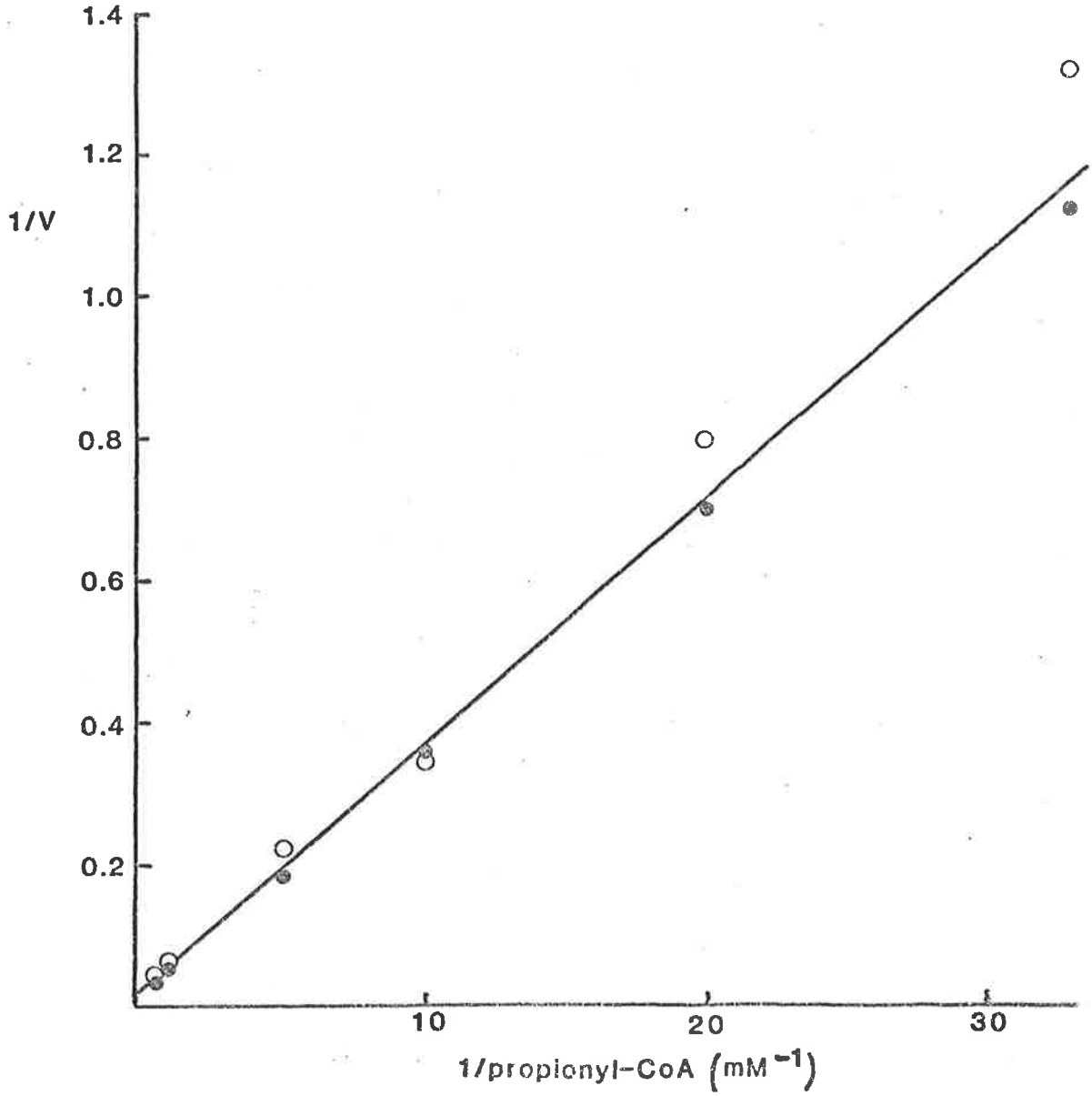
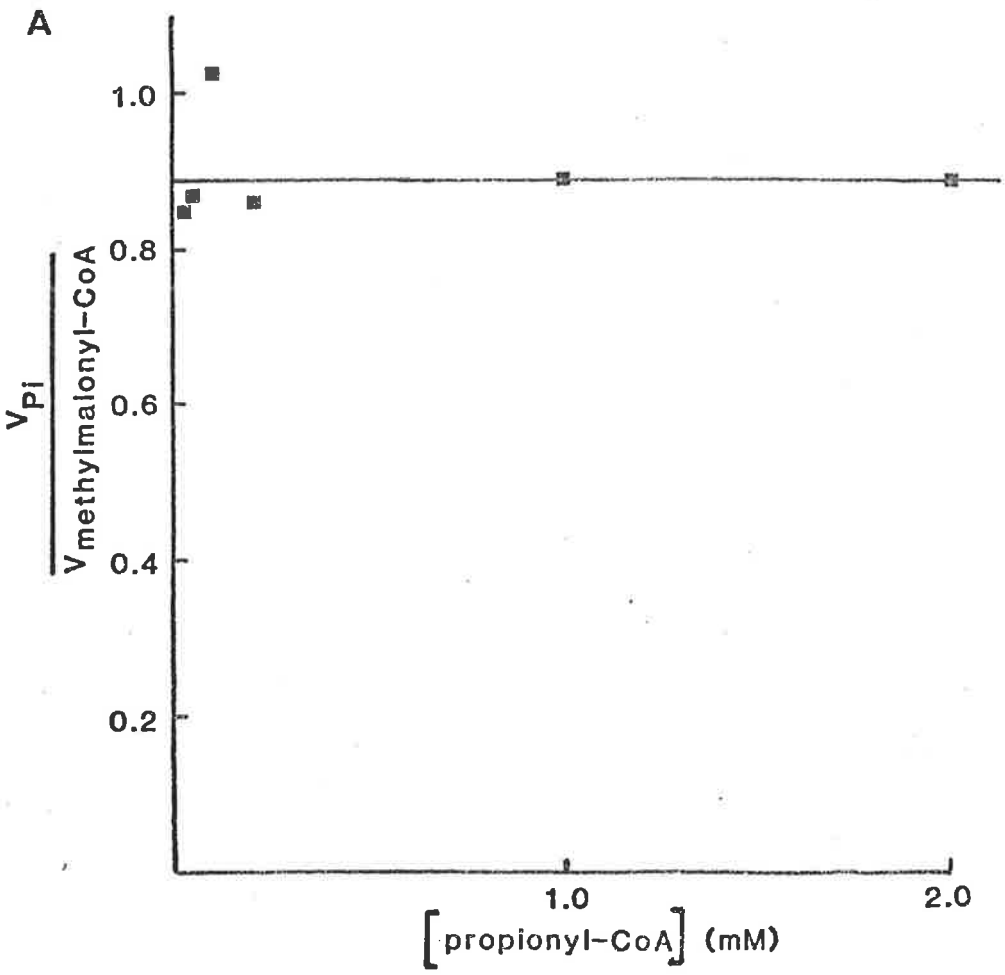


FIGURE 7.9

RECIPROCAL PLOTS OF PHOSPHATE AND METHYL-MALONYL-CoA PRODUCTION RATES AGAINST PROPIONYL-CoA CONCENTRATION

The reaction mixture contained 2.16 mM ATP, 7 mM $MgCl_2$, 2 mM DTE, 100 mM KCl, 25 mM $NaHCO_3$, 0.1 M N-ethylmorpholine-HCl buffer, pH 8.0, 0.002 units of propionyl-CoA carboxylase and the indicated amounts of propionyl-CoA in a volume of 0.2 ml. Pi release was measured by using [γ-^{32}P]ATP (○) and methylmalonyl-CoA by using $NaH^{14}CO_3$ (●), by the method described in Section 7.2.1.



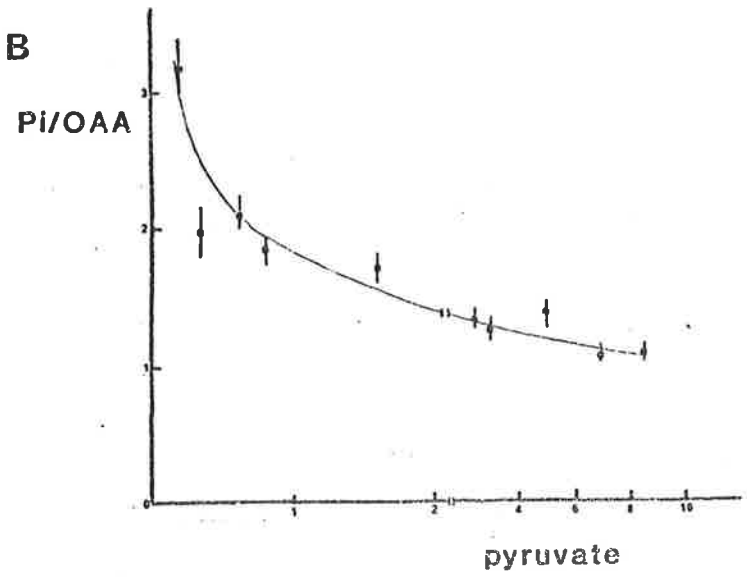


FIGURE 7.10

STOICHIOMETRY OF ATP HYDROLYSIS AND CO_2 FIXATION

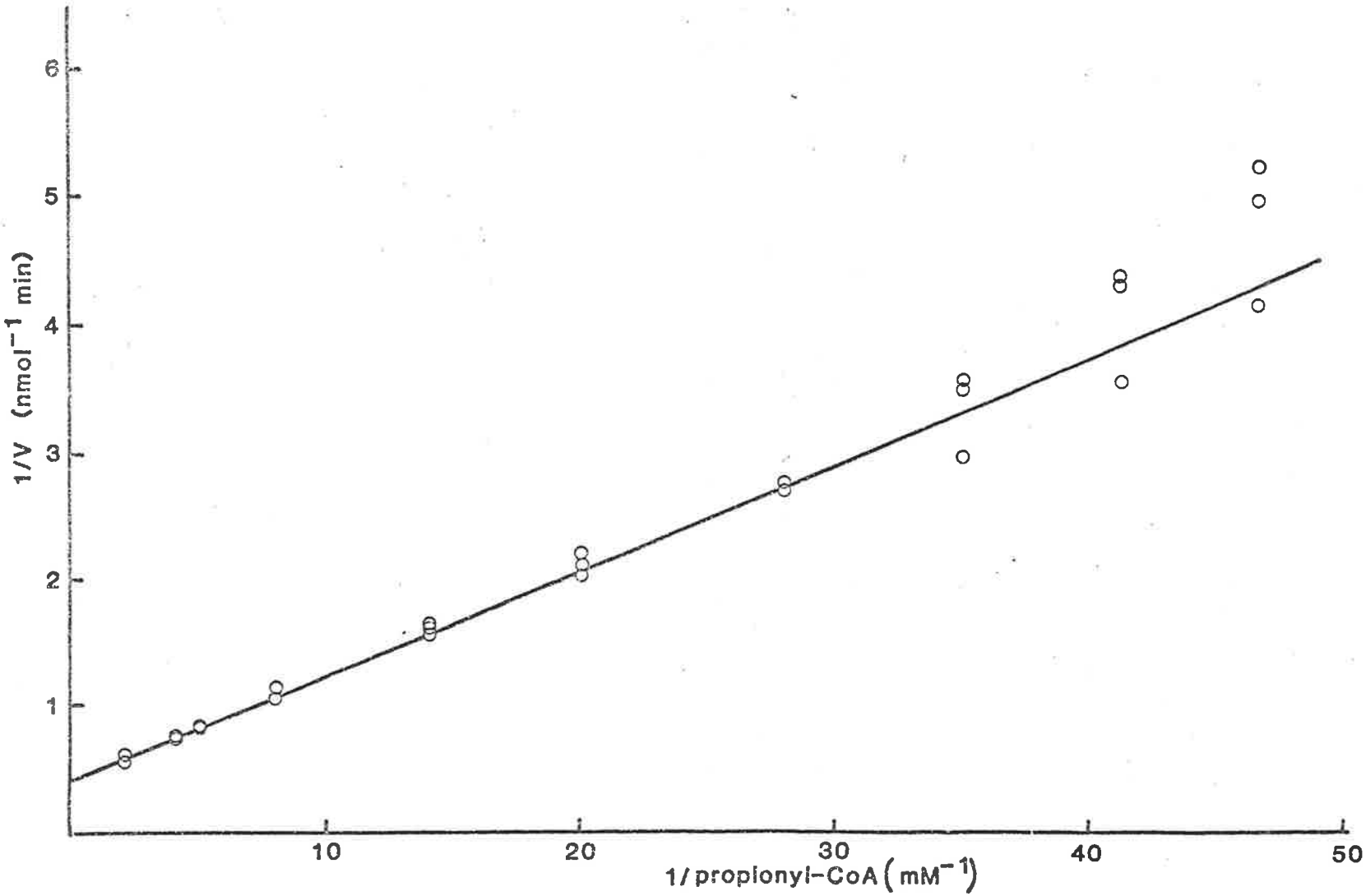
A. The ratio of Pi to methylmalonyl-CoA is plotted against propionyl-CoA concentration, using the data from Fig. 7.9.

B. The ratio of Pi to oxaloacetate produced by pyruvate carboxylase is plotted against pyruvate concentration. This figure is taken from Easterbrook-Smith *et al.* (1976a).

FIGURE 7.11

RECIPROCAL PLOT WITH VARYING PROPIONYL-CoA

The reaction solution contained 5 mM ATP, 10 mM $MgCl_2$, 100 mM KCl, 2 mM DTE, 25 mM $NaH^{14}CO_3$ (5 μCi/μmol), 0.1 M N-ethylmorpholine-HCl buffer, pH 8.0, 0.005 units of propionyl-CoA carboxylase and the indicated concentrations of propionyl-CoA, in a volume of 0.42 ml. The reaction was carried out under the conditions of the standard radiochemical assay (Section 2.2.6).


1/V (nmol^{-1} min)
6
5
4
3
2
1
10
20
30
40
50
1/propionyl-CoA (mM^{-1})

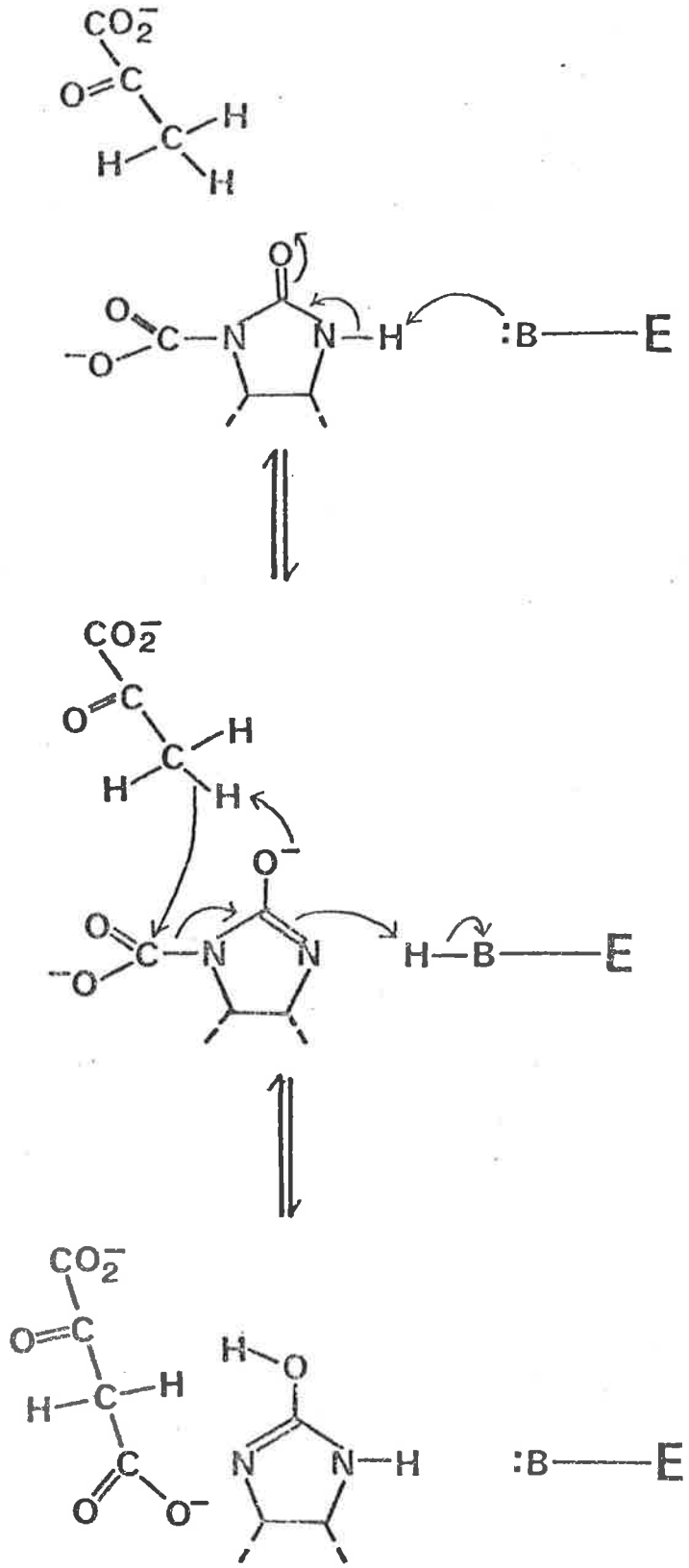


FIGURE 7.12

PROPOSED MECHANISM OF THE CARBOXYL GROUP TRANSFER REACTION

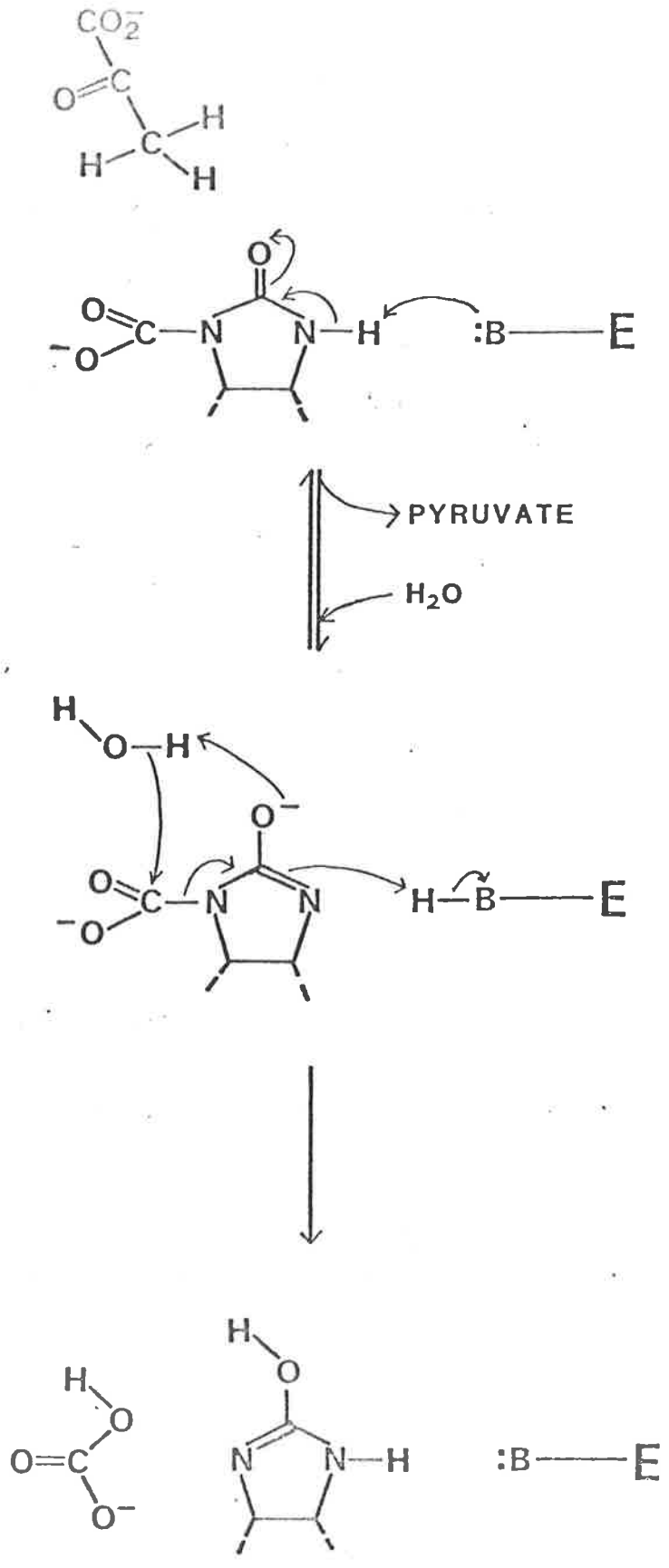
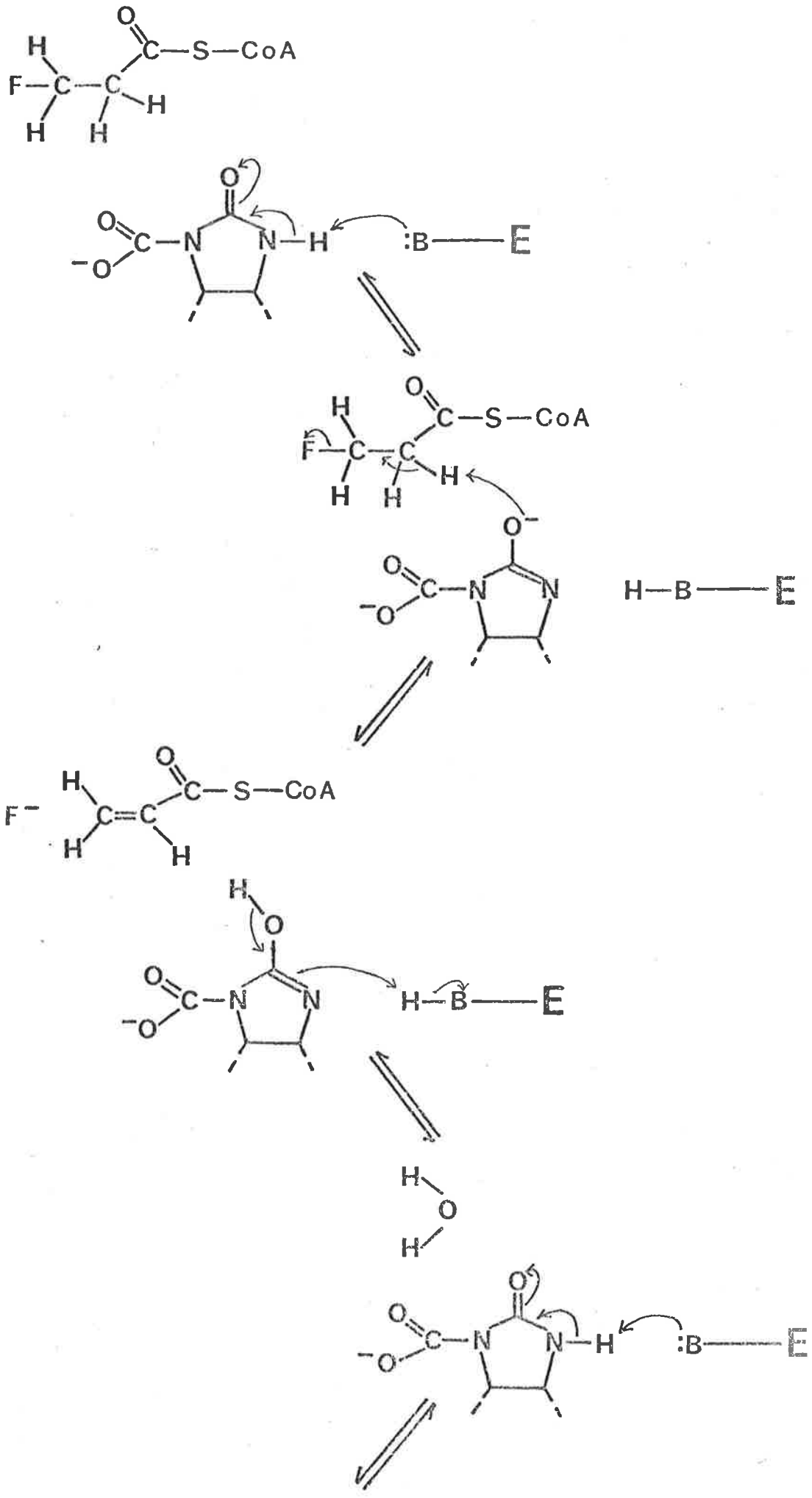


FIGURE 7.13

PROPOSED MECHANISM OF THE ABORTIVE DECARBOXYLATION PATHWAY.



Hydrolysis pathway (Fig. 7.13)

FIGURE 7.14 PROPOSED MECHANISM FOR THE ELIMINATION OF F^- FROM β-FLUOROPROPIONYL-CoA

CHAPTER 8

GENERAL DISCUSSION

The results presented in this thesis have been discussed within each chapter. In this chapter an overview of the project is presented and suggestions for further investigations are made.

8.1 THE EVOLUTION OF THE BIOTIN-DEPENDENT ENZYMES

The hypothesis that there is an evolutionary relationship between the biotin-dependent enzymes has formed a theme throughout the work described in this thesis. Such an hypothesis can rarely be proven or disproven, because only the present-day enzymes are available for study. Relatedness usually remains a matter of probability.

The amino acid composition comparisons described in Chapter 5 indicate that the biotin-dependent enzymes do not have a conserved sequence throughout most of the polypeptide chain in the way, for example, the serine proteases do. This does not invalidate the hypothesis, since proteins evolve by stepwise changes that can involve the insertion or deletion of sections of polypeptide or whole domains. Thus more definite information might be obtained by studying the parts of the molecules most likely to be conserved.

The need to interact productively with biotin may have been a conserving force during the evolution of these enzymes, in the same way that the need to bind NAD has maintained the dinucleotide binding domain typical of the dehydrogenases. Most of the biotin-dependent enzymes possess a specific acyl-CoA binding site and several have a specific ATP-binding site, so there may be several conserved domains, and any con-

servation of amino acid sequence is most likely to have been in the region of the binding sites for biotin, acyl-CoA and ATP. This could possibly be revealed by the isolation and sequencing of affinity labelled peptides. From the results of the amino acid composition comparisons, a comparison of peptides from pyruvate carboxylase and β-methylcrotonyl-CoA carboxylase would be a good starting point. The special case of the biotin attachment site has been discussed in Section 1.1.4. Work is underway in this laboratory on the isolation of a biotin-containing tryptic peptide from propionyl-CoA carboxylase.

In preliminary work with potential affinity labels on propionyl-CoA carboxylase, described in Chapter 4, labelling occurred at more than one location, indicating that it might not be a straightforward task to isolate unique peptides from the ATP and propionyl-CoA binding sites of this enzyme. A possible extension of this work would be to construct peptide maps of the modified α and β subunits to find the reason for the cross-protection by ATP and propionyl-CoA that was observed.

As both subunits were labelled by N-ethylmaleimide, oATP and o-dephosphopropionyl-CoA, it was suggested that both subunits contribute to the binding sites for ATP and propionyl-CoA. It is interesting that similar results have been obtained with succinyl-CoA synthetase. It has been shown that the active site of this enzyme is located at the interface between α and β subunits (Pearson & Bridger, 1975; Collier & Nishimura, 1978). When the periodate oxidation product of GDP was used as an affinity label, both subunits

were modified. The substrates GDP and CoA each protected both subunits to some extent (Ball & Nishimura, 1980). In contrast, however, affinity labels based on the CoA molecule-oxidized CoA disulphide and S-(4-bromo-2,3-dioxobutyl)-CoA - only modified the β subunit (Collier & Nishimura, 1978; Nishimura *et al.*, 1980).

Kinetic studies need to be done to establish whether o-dephosphopropionyl-CoA is a true affinity label of propionyl-CoA carboxylase, although the fact that less incorporation of the label occurs when propionyl-CoA is present suggests that it may be. Possible alternative affinity labels based on propionyl-CoA include S-(4-bromo-2,3-dioxobutyl)-CoA (Owens & Barden, 1978), 3-pentanoyl-CoA (Holland *et al.*, 1973), oxidized CoA disulphide (Collier & Nishimura, 1978) and p-azidobenzoyl-CoA (Lau *et al.*, 1977).

An unexpected finding from the amino acid composition comparisons was that within the proteins examined, the subunits of any one enzyme are more likely to be related to each other than to subunits of different enzymes. It would be interesting to see whether this applies to the rest of the biotin-dependent enzymes. Amino acid sequence information would be very useful in assessing the significance of the finding. Presumably, in each case the subunits arose by the duplication of an ancestral gene, followed by various different mutations in the daughter genes. This also seems to have happened during the course of the evolution of the acid proteases (Tang *et al.*, 1978). These enzymes are composed of two domains in a bilobal structure, with the active site in a cleft between the two domains. There are

some similarities in the amino acid sequences of the two domains, particularly at the active site, and there is a marked topological similarity throughout most of each domain. Tang *et al.* suggest a possible mechanism for the evolution of the acid proteases that involves gene duplication, divergence and gene fusion.

8.2 MECHANISM OF THE CARBOXYL GROUP TRANSFER REACTION

The experiments examining the factors that influence the location of carboxybiotin showed that Mg^{2+} inhibited the second partial reaction of pyruvate carboxylase and stabilized the ENZ-carboxybiotin complex against hydrolysis. The Mg^{2+} may hold the carboxybiotin in the first sub-site by co-ordinating directly with the carboxybiotin, since studies with free biotin and with model compounds indicate that metal ions can interact with the ureido group (Griesser *et al.*, 1973), the sulphur atom (Sigel *et al.*, 1969) and the 1-'N-carboxyl group (Tsuda *et al.*, 1979). It is not known whether the Mg^{2+} ion that inhibits the second partial reaction is the same as that which activates in the first partial reaction. The similarity between the dissociation constants obtained in the carboxyl transfer experiments (Chapter 6) and by Bais & Keech (1972) indicate that it may be the same ion. However, binding studies using $^{57}Co^{2+}$ show that more and more metal ions bind to pyruvate carboxylase as the concentration of metal ions increases (G.S. Baldwin, personal communication). The binding of the metal ions is decreased by low pH and by the presence of ethyl-CoA. Under

the conditions of the overall assay (pH 8.0, 0.25 mM ethyl-CoA) the enzyme monomer can bind at least 4 atoms of Co^{2+}.

The Mg^{2+} ion that activates the enzyme exerts its effect on the first partial reaction and so probably binds at the first sub-site. If the same Mg^{2+} also inhibits carboxybiotin translocation, then a treatment that preferentially damages the first sub-site (perhaps, for example, limited proteolysis) may decrease the inhibition by Mg^{2+} of the second partial reaction. The second partial reaction can be monitored by the pyruvate-oxaloacetate exchange reaction, which has been observed to be inhibited by Mg^{2+} (G.S. Baldwin & G.J. Goodall, unpublished observation). As the pyruvate-oxaloacetate exchange reaction is tedious to measure, it may be more convenient to measure the rate of release of ^3H from [3-^{3}H]pyruvate by pyruvate carboxylase in the presence of oxaloacetate. The reaction involved here would be the same as the pyruvate-oxaloacetate isotope exchange reaction, although it would be slower because of the tritium isotope effect. The rate of loss of ^3H from pyruvate could be easily measured by quenching the reaction with semicarbazide-hydrochloride, drying a sample to remove ^3H_2O, and counting the remaining radioactivity.

One obvious extension of the work on the transfer of the carboxyl group from isolated ENZ-carboxybiotin to 2-oxobutyrate would be to study the transfer of the carboxyl group to pyruvate, using a rapid quench apparatus. This might allow one or more rate constants to be determined.

Preliminary rapid quench experiments have already been performed in this laboratory. Such experiments can also be carried out on propionyl-CoA carboxylase, either using propionyl-CoA as substrate in a rapid quench apparatus, or perhaps using a poor substrate such as butyryl-CoA or acetyl-CoA and the method developed for pyruvate carboxylase with 2-oxobutyrate. Preliminary experiments indicate that propionyl-CoA carboxylase, like pyruvate carboxylase, is inhibited by high concentrations of Mg^{2+}, but the enzymes differ in that Mg^{2+} increases the rate of hydrolysis of the isolated propionyl-CoA carboxylase-carboxybiotin complex. Further studies of such differences between the two enzymes may help delineate the essential from the fortuitous features of biotin-dependent carboxyl group transfer reactions.

Another possible extension to the study of the carboxyl group transfer reaction lies in the identification of the base that was proposed to remove the 3'-N proton from carboxybiotin. This base must be unprotonated when carboxybiotin binds at the second sub-site, and so a likely candidate is histidine. However, a base at the active site of an enzyme can have a significantly perturbed pK value, so that a single pH profile is insufficient to identify a base involved in catalysis. Cleland (1977) has reviewed the methods available for identifying catalytic groups by pH-variation studies.

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PUBLICATIONS

Factors that influence the translocation of the N-carboxybiotin moiety between the two sub-sites of pyruvate carboxylase

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